PERFORMANCE OF THE NOVEL PARTEC RAPID MALARIA TEST® IN THE DIAGNOSIS OF MALARIA IN A RURAL ENDEMIC AREA; A QUICKER, CHEAPER AND COST EFFECTIVE ALTERNATIVE?

by

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DECLARATION

I hereby declare that this submission is my own work towards the award of an MPhil Degree and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

I dedicate
this work to
my lovely wife and son.

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

GS: Giemsa stained microscopy

PM: Partec Rapid Malaria Test$^{®}$

BN RDT: Binax Now$^{®}$ Rapid Diagnostic Test

PCR: Polymerase Chain Reaction

RBC: Red Blood cell

WBC: White Blood cell

HPF: High Power Field

QBC: Quantitative Buffy Coat

KAO: Kawamoto Acridine Orange

BCP: Benzothiocarboxypurine

PPV: Positive Predictive Value

NPV: Negative Predictive Value

ABSTRACT

Malaria remains the single largest cause of death in Africa killing one child in every 30 seconds but treatment decisions are often made based on clinical diagnosis since laboratory techniques to confirm the clinical suspicion are labour-intensive and costly. This study evaluated the recently developed Partec Rapid Malaria Test® (PM) for the detection of *Plasmodium spp.* in human blood from patients in an endemic area and compared the results with thick blood film Giemsa stain (GS), Binax NOW® rapid diagnostic test (BN RDT) and Real time PCR in terms of their performance and operational characteristics using an expanded reference as the gold standard. A total of seven hundred and fifty one (751) participants were involved in this study. Out of this 400(53.2%) were males and 351(46.7%) were females. Their ages ranged from 3 to 16 years with the modal age being 4 years (150/752).Using an expanded reference standard the sensitivities of GS, PM, Real time PCR and BN RDT were 96.7%, 97.3%, 97.3% and 96.5% respectively. The specificities were 100%, 98.5%, 64.6% and 96.7% respectively. The Positive Predictive Values (PPV) for GS, PM, Real time PCR and BN RDT were 100%, 97.7%, 62.8% and 95.6% respectively whilst the Negative Predictive Values (NPV) were 97.8%, 98.2%, 97.5% and 97.3% respectively. There was a strong agreement between three tests methods and the reference standard: k=0.97, 0.96 and 0.93 respectively for GS, PM and BN RDT but not with Real time PCR (k=0.56). Compared to each other, the tests methods had a strong agreement as well: GS vs PM, k= 0.96 and for PM vs BN RDT, k=0.87. Real time PCR had a higher positive detection rate compared to the other methods; 110/488 (22.5%) and 109/488 (22.3%) of the total samples were positive for Real time PCR but not GS and PM respectively. Parasite counts obtained from the PM were relatively lower than that obtained from the GS but the PM had better operational characteristics than the GS. The PM may therefore be used as an alternative method for Giemsa thick film staining but for parasite speciation, the Giemsa thin film remains preferable.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is the most common single diagnosis made in most countries in Africa (WHO, 2003) . However, in many endemic countries, clinical diagnosis is the only method used to decide on treatment even though its accuracy is limited by the low specificity of signs and symptoms of malaria (Chandramohan *et al.*, 2002; Källander *et al.*, 2004; Mwangi *et al.*, 2005). Presumptive antimalaria treatment for any fever with no obvious alternative cause is widely practiced, and studies suggest that this leads to significant overuse of antimalaria drugs throughout Africa (Amexo *et al.*, 2004; Barat *et al.*, 1999; Mwangi *et al.*, 2005). This over-diagnosis of malaria in the formal healthcare sector coexists with under-diagnosis of malaria in the community, with the result that antimalaria drugs are given to people who do not need them and not given to those who do (Mutabingwa *et al.*, 2005; White *et al.*, 1999). This practice results in the erroneous treatment of fever caused by other infections, and may exert a high drug pressure on malaria parasite populations, leading to selection of drug-resistant parasites(Lema *et al.*, 1999).

Reliable diagnosis of malaria requires laboratory confirmation of the presence of malaria parasites in the blood of a febrile patient (Nevill, 1990), hence a prompt and accurate diagnosis of malaria is the key to effective disease management. Laboratory confirmation of malaria infection requires the availability of a rapid, sensitive, and specific test at an affordable cost. However, in many endemic countries, laboratory techniques to confirm the clinical diagnosis of malaria are considered to be too labour-intensive (Bojang et al.*,* 2000) and unreliable due to lack of skilled

microscopists, limited supplies, inadequate maintenance of microscopes and reagents, and inadequate or absence of quality control systems (Coleman et al., 2002).

In general, the screening of blood slides by Giemsa stained microscopy is still considered as the gold standard (Jonkman *et al.*, 1995). This method is cheap and simple but labour-intensive, time-consuming and requires well-trained personnel (Reyburn *et al.*, 2004) particularly at low parasite levels (Coleman *et al.*, 2002). In resource-poor areas, microscopic diagnosis has been shown to be insensitive and nonspecific, especially when parasitaemia is low or mixed infections are present (Amexo *et al.*, 2004; Coleman *et al.*, 2002). Under optimal conditions, the sensitivity of thick film microscopy is 10–30 parasites per micro liter (μl) of blood (Gilles, 1993); sensitivities and specificities as low as 71–72% have been reported (Arai *et al.*, 1996; Snounou *et al.*, 1993a) thus, the availability of a simple and accurate test that requires little or no training and with high sensitivity and specificity could greatly aid the diagnosis of malaria infection in remote areas where health facility coverage is low and the populations are at high risk of contracting malaria (Moody, 2002; WHO, 2000) .

Over the years, alternative methods of malaria diagnosis have been introduced to overcome the limitations of conventional microscopy. Some of these methods include: Rapid Diagnostic Tests (RDT), Fluorescence Microscopy, Molecular Techniques such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence-based Amplification (NASBA), Flow Cytometry, Mass Spectrometry and Malaria Magnetic Deposition Microscopy (MDM) (Zimmerman *et al.*, 2006).

1.2 Problem Statement

One of the main interventions of the Global Malaria Control Strategy is the prompt and accurate diagnosis of the disease as it is the key to effective disease management (WHO, 1993). It is thus of concern that poor diagnosis continue to hinder effective malaria control. This is due to a combination of factors, including non-specific clinical presentation of the disease, high prevalence of asymptomatic infection in some areas, lack of resources and insufficient access to trained health care providers and health facilities, and widespread practice of self-treatment for clinically suspected malaria (WHO, 2000). One major contributing factor, however, is that the laboratory diagnosis of malaria has up till now relied nearly exclusively on Giemsa stained microscopy, a valuable technique when performed correctly but it is unreliable and wasteful when poorly executed. A better utilization of microscopy and the development of alternative diagnostic techniques could substantially improve malaria control (Cook, 1992). Such objectives prove particularly relevant to the Roll Back Malaria initiative, a global movement that emphasizes better application of existing tools and the development of new ones (WHO, 2000).

1.3 Aim

The aim of this study is to evaluate the recently developed Partec Rapid Malaria Test[®] (PM) (Partec GmbH, Münster, Germany) for the detection of *Plasmodium spp* in human blood (from patients in an endemic area) and compare the results with other techniques such as Real time PCR, Binax NOW[®] RDT (BN RDT) and Giemsa stain (GS) using an Expanded Reference Standard (Thejls *et al.*, 1994).

1.4 Objectives

The specific objectives of this study are:

- To compare the test performance i.e. sensitivity, specificity, positive and negative predictive values of the GS, PM, Real time PCR and BN RDT using an Expanded Reference Standard (Thejls *et al.*, 1994).

  - To assess the agreement or otherwise between the four different techniques (GS, PM, Real time PCR and BN RDT).

  - Compare operational characteristics such as labour, time, cost-effectiveness and ease of use of the PM to the other methods.

  - Offer suggestions for the use of these tests in the diagnosis of malaria.

1.5 Justification

Malaria remains the single largest cause of death in Africa, where it kills one child in every 30 seconds (GFHR, 2006), this translates to the deaths of approximately 3000–6000 children a day (Amexo *et al.*, 2004). The World Health Organization estimates 300-500 million cases of malaria infections resulting in over one million deaths occurring globally annually (Filler *et al.*, 2003). Rapid and accurate diagnosis is therefore the cornerstone of good malaria control. Initiation of malaria treatment largely depends on good laboratory-confirmed diagnosis. Microscopy is generally available at district hospitals but it is either not used (Källander *et al.*, 2004)or has low accuracy (El-Nageh, 1996). Microscopy depends on well-maintained equipment, uninterrupted supply of good-quality reagents, trained staff, and good quality monitoring and supervisory systems. Maintaining a quality-assured microscopy service is a major challenge even for district hospitals, so microscopy is not suitable for routine use at community level (Amexo *et al.*, 2004; Dowling *et al.*, 1966) Accurate diagnosis therefore requires specific and good quality diagnostic tests that

are fit for purpose and provides accurate results (Peeling *et al.*, 2008). Giemsa stained thick and thin film microscopy is still the method of choice for the diagnosis of malaria in endemic areas because it is an inexpensive and an easy method. At best, the sensitivity of detection by microscopy is approximately 10–30 parasites/µl of blood (Gilles, 1993). The presumptive treatment of fever as malaria has resulted in the over-administration of antimalaria drugs (Snounou *et al.*, 1993a) which has in turn led to the erroneous treatment of fever caused by other infections thus has exerted a high drug pressure on malaria parasite populations, leading to selection of drug-resistant parasites. A key to effective management of malaria and its complications is prompt and accurate diagnosis thus the need to evaluate the various techniques, compare and assess their effectiveness in the diagnosis of malaria and whether patients can afford them.

1.6 Hypothesis

- Recently developed Partec rapid malaria test[®] is a superior diagnostic tool to the Giemsa stain for malaria diagnosis in rural endemic areas
- The Partec rapid malaria test[®] and the Binax Now[®] rapid diagnostic test can be used as point-of-care tests in malaria endemic areas.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of malaria

Malaria was once thought to be caused by breathing in foul swamp vapour: the name is from the Italian word "mal" - bad - and "aria", air (Suh *et al.*, 2004). Swamps are indeed a cause, because they are breeding grounds for mosquitoes, which spread the malaria parasite from person to person through their bite (Suh *et al.*, 2004). Towards the end of the 19th century, Charles Louis Alphonse Laveran, a French army surgeon, noticed parasites in the blood of a patient suffering from malaria and Dr Ronald Ross (Tuteja, 2007), a British medical officer in Hyderabad, India, discovered that mosquitoes transmitted malaria. The Italian professor Giovanni Battista Grassi subsequently showed that human malaria could only be transmitted by *Anopheles* mosquitoes (Tuteja, 2007).

2.1.1 The parasite

Malaria is transmitted through the bite of an infected female *Anopheles* mosquito. Of the approximately 400 species of *Anopheles* throughout the world, about 60 are malaria vectors under natural conditions, 30 of which are of major importance (Tuteja, 2007). Malaria parasites are eukaryotic single-celled microorganisms that belong to the genus *Plasmodium* (Tuteja, 2007). Only 4 of the species of plasmodia are infectious to humans (Tuteja, 2007). The majority of cases and almost all deaths are caused by *Plasmodium falciparum* (Snow *et al.*, 2004). *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* cause less severe disease (Suh *et al.*, 2004). These four species differ morphologically, immunologically, in their geographical distribution, in their relapse patterns and in their drug responses (Tuteja,

2007). *P. falciparum* is the agent of severe, potentially fatal malaria and is the principal cause of malaria deaths in young children in Africa (Snow *et al.*, 2004) and generally 90% of all cases in Africa (Suh *et al.*, 2004).

2.1.2 Parasite Distribution

Malaria is endemic in 109 countries and is found throughout the tropics (WHO, 2008b) (Figure 1). In Africa, *P. falciparum* predominates, as it does in Papua New Guinea and Haiti, whereas *P. vivax* is more common in Central and parts of South America, North Africa, the Middle East and the Indian subcontinent (Cook *et al.*, 2008). The prevalence of both species is approximately equal in other parts of South America, South-east Asia and Oceania. *P. vivax* is rare in sub-Saharan Africa (except for the horn of Africa), whereas *P. ovale* is common only in West Africa (Cook *et al.*, 2008). *P. malariae* is found in most areas, but it is relatively uncommon outside Africa. Malaria was once endemic in Europe and northern Asia and was introduced to North America but it has been eradicated from these areas. In northern China and North Korea, *P. vivax* strains (*P. vivax* hibernans) with long incubation periods and long intervals (10–12 months) between relapses may still be found (Cook *et al.*, 2008).
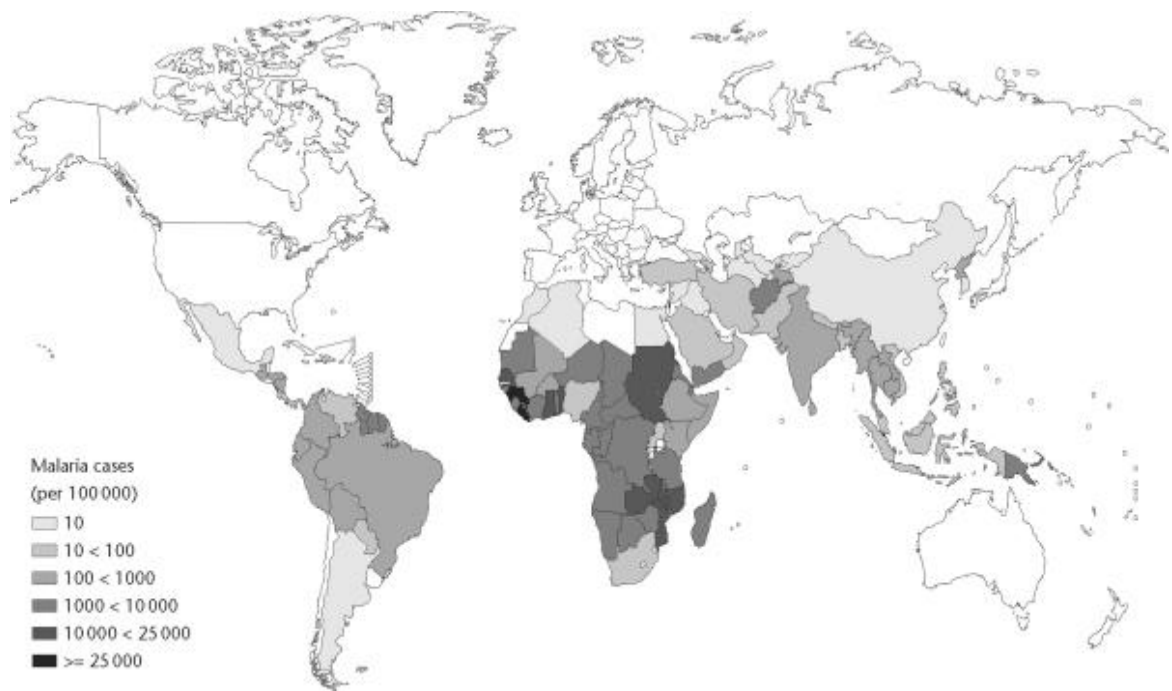
Figure 1. Global distribution of malaria. WHO map.

Source: http://gamapserver.who.int/mapLibrary/Files/Maps/global_cases.jpg

2.1.3 Life cycle of the malaria parasite

The life cycle of malaria parasites is extremely complex and requires specialized protein expression for survival in both the invertebrate and vertebrate hosts (Tuteja, 2007). These proteins are required for both intracellular and extracellular survival, for the invasion of a variety of cell types and for the evasion of host immune responses (Tuteja, 2007). Once injected into the human host, *P. falciparum* and *P. malariae* sporozoites trigger immediate schizogony, whereas *P. ovale* and *P. vivax* sporozoites may either trigger immediate schizogony or lead to delayed schizogony as they pass through the hypnozoite stage. The life cycle of the malaria parasite is shown in Figure 2 and can be divided into several stages, starting with sporozoite entry into the bloodstream.

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2.1.3.1 Tissue schizogony (pre-erythrocytic schizogony)

Infective sporozoites from the salivary gland of the *Anopheles* mosquito are injected into the human host along with anticoagulant-containing saliva to ensure an even-flowing blood meal (Tuteja, 2007). It was thought that sporozoites move rapidly away from the site of injection, but a recent study using a rodent parasite species (*Plasmodium yoelii*) as a model system indicates that the majority of infective sporozoites remain at the injection site for hours, with slow release into the circulation (Yamauchi *et al.*, 2007). Once in the human bloodstream, *P. falciparum* sporozoites reach the liver and penetrate the liver cells (hepatocytes) where they remain for 9-16 days and undergo asexual replication known as exo-erythrocytic schizogony. The mechanism of targeting and invading the hepatocytes is not yet well understood, but studies have shown that sporozoite migration through several hepatocytes in the mammalian host is essential for completion of the life cycle (Mota *et al.*, 2001). The receptors on sporozoites responsible for hepatocyte invasion are mainly the thrombospondin domains on the circumsporozoite protein and on thrombospondin-related adhesive protein (Miller *et al.*, 2002). These domains specifically bind to heparin sulfate proteoglycans on the hepatocytes (Frevert *et al.*, 1993). Each sporozoite gives rise to tens of thousands of merozoites inside the hepatocyte and each merozoite can invade a red blood cell (RBC) on release from the liver. In an interesting study, also using rodent malaria parasites (*Plasmodium berghei*), it has been shown that liver-stage parasites manipulate their host cells to guarantee the safe delivery of merozoites into the bloodstream (Sturm *et al.*, 2006). Hepatocyte-derived merosomes appear to act as shuttles that ensure the protection of parasites from the host immune system and the release of viable merozoites directly into the circulation (Sturm *et al.*, 2006). The time taken to complete the tissue phase varies, depending on

the infecting species; (8-25 days for *P. falciparum*, 8-27 days for *P. vivax*, 9-17 days for *P. ovale* and 15-30 days for *P. malariae*) and this interval is called the prepatent period.

2.1.3.2 Erythrocytic schizogony

Merozoites enter erythrocytes by a complex invasion process, which can be divided into four phases: (a) initial recognition and reversible attachment of the merozoite to the erythrocyte membrane; (b) reorientation and junction formation between the apical end of the merozoite (irreversible attachment) and the release of substances from the rhoptry and microneme organelles, leading to formation of the parasitophorous vacuole; (c) movement of the junction and invagination of the erythrocyte membrane around the merozoite accompanied by removal of the merozoite's surface coat; and (d) resealing of the parasitophorous vacuole and erythrocyte membranes after completion of merozoite invasion (Miller *et al.*, 2002). This is because the invasion of erythrocytes by *P. falciparum* requires a series of highly specific molecular interactions, it is regarded as an attractive target for the development of interventions to combat malaria (Frevert *et al.*, 1993). Asexual division starts inside the erythrocyte and the parasites develop through different stages therein (Tuteja, 2007). The early trophozoite is often referred to as the ring form, because of its characteristic morphology (Tuteja, 2007) (Figure 3). Trophozoite enlargement is accompanied by highly active metabolism, which includes glycolysis of large amounts of imported glucose, the ingestion of host cytoplasm and the proteolysis of hemoglobin into constituent amino acids (Tuteja, 2007). Malaria parasites cannot degrade the heme by-product and free heme is potentially toxic to the parasite (Tuteja, 2007). Therefore during hemoglobin degradation, most of the

liberated heme is polymerized into hemozoin (malaria pigment), a crystalline substance that is stored within the food vacuoles (Miller *et al.*, 2002).

The end of this trophic stage is marked by multiple rounds of nuclear division without cytokinesis resulting in the formation of schizonts (Miller *et al.*, 2002) (Figure 3). Each mature schizont contains around 20 merozoites and these are released after lyses of the red blood cells (RBC) to invade further uninfected RBCs. This release coincides with the sharp increases in body temperature during the progression of the disease (Tuteja, 2007). This repetitive intraerythrocytic cycle of invasion multiplication release invasion continues, taking about 48 h in *P. falciparum*, *P. ovale* and *P. vivax* infections and 72 h in *P. malariae* infection (Miller *et al.*, 2002; Tuteja, 2007). It occurs quite synchronously and the merozoites are released at approximately the same time of the day (Tuteja, 2007). The contents of the infected RBC that are released upon its lyses stimulate the production of tumor necrosis factor and other cytokines, which are responsible for the characteristic clinical manifestations of the disease. A small proportion of the merozoites in the red blood cells eventually differentiate to produce micro and macrogametocytes (male and female, respectively), which have no further activity within the human host (Carter *et al.*, 1980). These gametocytes are essential for transmitting the infection to new hosts through female *Anopheles* mosquitoes (Carter *et al.*, 1980). Normally, a variable number of cycles of asexual erythrocytic schizogony occur before any gametocytes are produced (Tuteja, 2007). In *P. falciparum*, erythrocytic schizogony takes 48 h and gametocytogenesis takes 10-12 days (Tuteja, 2007). Gametocytes appear on the fifth day of primary attack in *P. vivax* and *P. ovale* infections, and thereafter become numerous; they appear at anytime from 5-23 days after a primary attack by *P. malariae* (Tuteja, 2007).

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2.1.3.3 Sexual phase in the mosquito (sporogony)

A mosquito taking a blood meal on an infected individual may ingest these gametocytes into its midgut, where macrogametocytes form macrogametes and exflagellation of microgametocytes produce microgametes (Tuteja, 2007). These gametes fuse, undergo fertilization and form a zygote. This transforms into an ookinete, which penetrates the wall of a cell in the midgut and develops into an oocyst (Tuteja, 2007). In a recent study, it has been shown that gamete surface antigen Pfs230 mediates human RBC binding to exflagellating male parasites to form clusters termed exflagellation centers, from which individual motile microgametes are released. This protein thus plays an important role in subsequent oocyst development, which is a critical step in malaria transmission (Eksi *et al.*, 2006). Sporogony within the oocyst produces many sporozoites and when the oocyst ruptures, they migrate to the salivary glands for onward transmission into another host. This form of the parasite is found in the salivary glands after 10-18 days and thereafter the mosquito remains infective for 1-2 months. When an infected mosquito bites a susceptible host, the *Plasmodium* life cycle begins again (Tuteja, 2007).
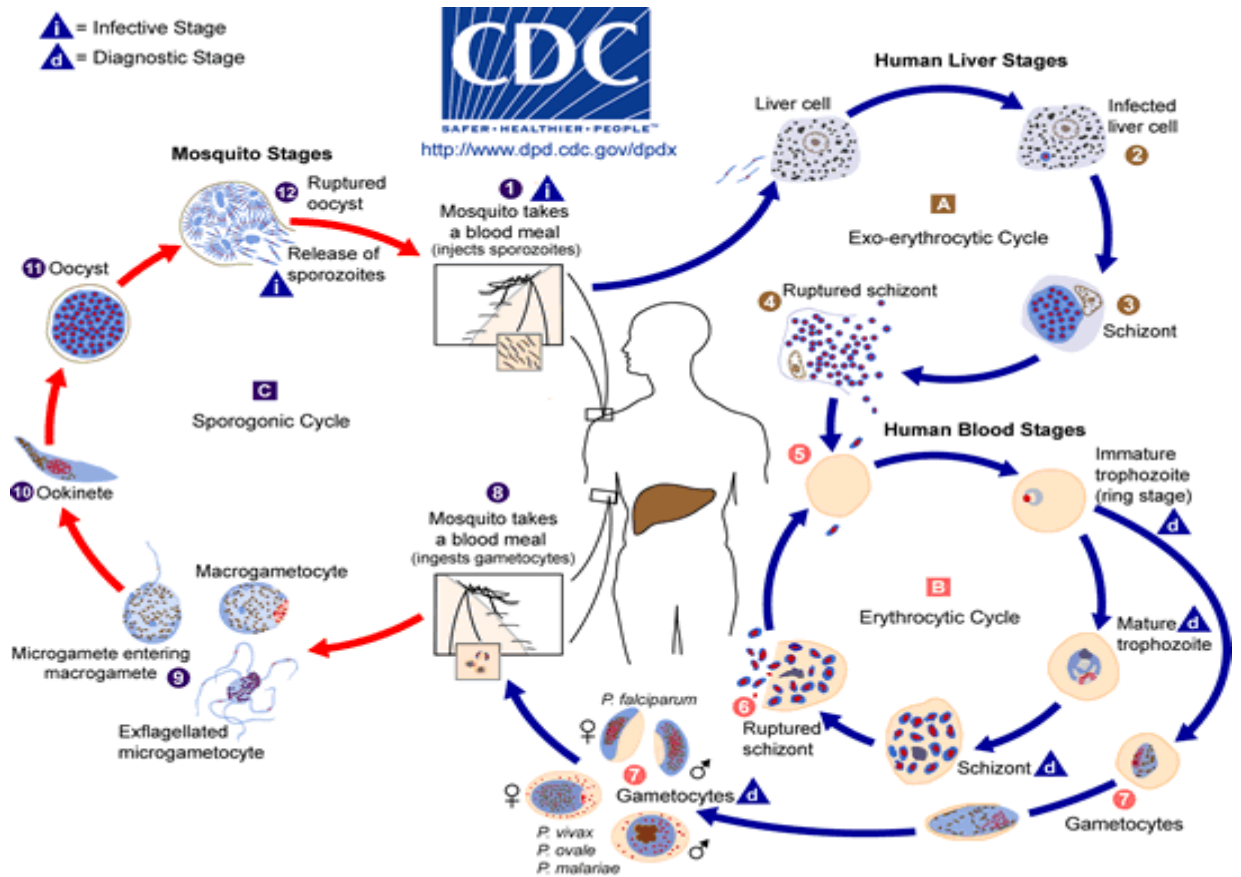
Figure 2: Schema of the Life Cycle of Malaria.

Source: http://www.cdc.gov/malaria/biology/life_cycle.htm
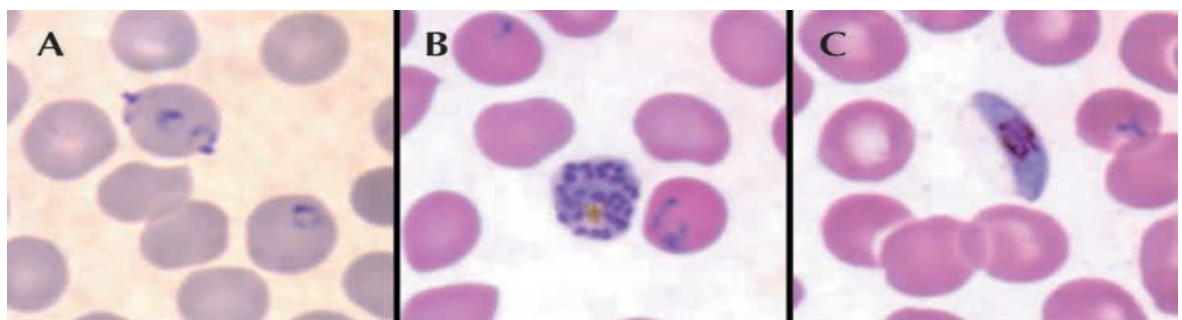
Figure 3: Stages in the life cycle of *Plasmodium falciparum*. **A:** Ring forms (early trophozoites). **B:** Mature schizont, rarely seen in peripheral blood smears because of microvascular sequestration. **C:** Gametocyte, demonstrating the classic banana shape.

Source: Suh et al., 2004

2.2 Diagnosis of malaria

The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wrights, or Fields stain (Warhurst *et al.*, 1996). However in resource-poor areas, microscopic diagnosis has been shown to be insensitive and non-specific, especially when parasitaemia is low or mixed infections are present (Amexo *et al.*, 2004; Coleman *et al.*, 2002). In field conditions, sensitivities and specificities as low as 71–72% have been reported (Snounou *et al.*, 1993a). Other limitations include false negativity due to relatively small amount of blood examined or low parasitaemia, and false positivity due to debris (Salako *et al.*, 1999). In an attempt to enhance the detection of malaria parasites, alternative methods have been introduced.

2.2.1 Rapid Diagnostic Tests (RDTs)

Malaria rapid diagnostic tests, sometimes called "dipsticks" or malaria rapid diagnostic devices (MRDDs) are simple immunochromatographic tests that identify specific antigens of malaria parasites in whole or peripheral blood (Hopkins *et al.*, 2008). RDTs are available as a simple dipstick, a cassette (dipstick in a plastic holder), or in a card format (Figure 4). Simplicity of format (e.g. cassettes) may be important to overall sensitivity (Tavrow *et al.*, 2000). A summary of selected RDTs with their target antigens and test performance can be seen in table 1 below (Page 18).

Dipstick   Cassette   Card

Figure 4: Examples of RDT formats.

Source: Dipstick and Cassette (MDCoE, Kisumu, Kenya); Card (Original)

2.2.1.1 Target Antigens

Malaria antigens currently targeted by RDT are Histidine-rich protein II of *P. falciparum* (PfHRPII), *Plasmodium* aldolase and Parasite lactate dehydrogenase (pLDH).

Histidine-rich protein II of *P. falciparum* (PfHRPII) is a water soluble protein that is produced by the asexual stages and gametocytes of *P. falciparum,* expressed on the red cell membrane surface, and shown to remain in the blood for at least 28 days after the initiation of antimalaria therapy. Several RDTs targeting PfHRP II have been developed (Rock *et al.*, 1987; Verle *et al.*, 1996).

Plasmodium aldolase is an enzyme of the parasite glycolytic pathway expressed by the blood stages of *P. falciparum* as well as the non-fa1ciparum malaria parasites. Monoclonal antibodies against *Plasmodium* aldolase are pan-specific in their reaction and have been used in a combined 'P.f/P.v' immunochromatographic test that targets the pan malarial antigen (PMA) along with Pf HRPII (Meier *et al.*, 1992; Miller *et al.*, 1994).

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Parasite lactate dehydrogenase (pLDH) is a soluble glycolytic enzyme produced by the asexual and sexual stages of the live parasites and it is present in and released from the parasite infected erythrocytes. It has been found in all 4 human malaria species, and different isomers of pLDH for each of the 4 species exist (Piper *et al.*, 1999). With pLDH as the target, a quantitative immunocapture assay, a qualitative immunochromatographic dipstick assay using monoclonal antibodies, an immunodot assay, and a dipstick assay using polyclonal antibodies have been developed.

2.2.1.2 Test performance of RDTs

2.2.1.2.1 Dipstick RDTs

Dipsticks are the commonly used forms of RDTs. This may be due to the fact that they are readily available in the market at a cheap price US$ 0.5/test (Guthmann *et al.*, 2008). They are easy and quick to use and thus require very little training and a shorter turn-around time. Dipsticks do not offer enough protection against blood contamination (Kakkilaya, 2003), but if protective gloves are used during testing procedures, this problem can be solved or minimized. Most formats detect only the HRPII antigen which is specific for *P. falciparum* and therefore areas where non falciparum malaria is predominant may not find this type of RDT formats very useful (Moody, 2002).

2.2.1.2.2 Cassette and Cards RDTs

These RDT formats are much safer to use. This is because they prevent blood contamination. They are also readily available but at a 40% price higher than the dipsticks (WHO, 2004). These tests are not as simple as the dipsticks and thus require proper training before use and they also require much time for results to be ready. Unlike the dipsticks, most of these RDT formats target two antigens (HRPII/Pan

pLDH, HRPII/Pan aldolase or HRPII/pLDH) (Moody, 2002) making it possible to detect all the plasmodium species.

2.2.2 Fluorescence Microscopy

The fluorescence microscope is based on the phenomenon that certain materials emit energy detectable as visible light when irradiated with light of a specific wavelength. The sample can either be fluorescing in its natural form like chlorophyll and some minerals, or treated with fluorescing chemicals (Moody, 2002). In malaria diagnosis, many methods have been developed based on this technique. Some of these methods are the Quantitative Buffy-Coat (QBC) method which is available as a commercial kit (QBC[®]; Becton Dickinson, Franklin Lakes, NJ); the Kawamoto Acridine-Orange (KAO) process (Kawamoto, 1991; Kong et al., 1995; Bosch *et al*., 1996), the Benzothiocarboxypurine (BCP) procedure (Makler *et al.*, 1998) and recently the Partec Rapid Malaria Test[®] (Partec GmbH, Münster, Germany).

Table 1: Test performance of some RDT formats

Brand	Format	Target Ag(s)	Species ID	Sensitivities (%)	Specificities (%)
Paracheck®	Dipstick and Cassette	HRPII	Pf only	100 (Swarthout *et al.*, 2007)	52 (Swarthout *et al.*, 2007)
				93 (Singh *et al.*, 2005b)	84 (Singh *et al.*, 2005b)
				90.1 (van den Broek *et al.*, 2006)	99.5 (van den Broek *et al.*, 2006)
OptiMAL®	Cassette	HRPII, pLDH or Pan aldolase	All species	89 (Iqbal *et al.*, 2003)	99 (Iqbal *et al.*, 2003)
				88.5 (Jelinek *et al.*, 1999)	99.4 (Jelinek *et al.*, 1999)
				76.2 (Grobusch *et al.*, 2003)	99.7% (Grobusch *et al.*, 2003)
				83.6 for Pf , (van den Broek *et al.*, 2006)	98.3 for Pf, (van den Broek *et al.*, 2006)
				91 for Pv (van den Broek *et al.*, 2006)	98.6 for Pv (van den Broek *et al.*, 2006)
ICT Malaria®	Card	HRPII	Pf only	82 (Iqbal *et al.*, 2003)	99.5 (Iqbal *et al.*, 2003)
				95.7 (Grobusch *et al.*, 2003)	99.2 (Grobusch *et al.*, 2003)
				92.5 (Jelinek *et al.*, 1999)	98.3 (Jelinek *et al.*, 1999)
Binax NOW®	Card	HRPII and pLDH	Pf and Pv	100 for Pf (Wongsrichanalai *et al.*, 2003)	96 for Pf (Wongsrichanalai *et al.*, 2003)
				87.3 for Pv (Wongsrichanalai *et al.*, 2003)	97.7 for Pv (Wongsrichanalai *et al.*, 2003)
				94 (Farcas *et al.*, 2003)	99 (Farcas *et al.*, 2003)
				90.8 for Pf (van den Broek *et al.*, 2006)	90.6 for Pf (van den Broek *et al.*, 2006)
				81.4 for Pv (van den Broek *et al.*, 2006)	99.4 for Pv (van den Broek *et al.*, 2006)
ParaSight-F®	Dipstick	HRPII	Pf only	90–92 (Lema *et al.*, 1999)	99 (Lema *et al.*, 1999)
				95.1 (Grobusch *et al.*, 2003)	97.1(Grobusch *et al.*, 2003)
				95 (Forney *et al.*, 2001)	86 (Forney *et al.*, 2001)
MAKROmed®	Dipstick	HRPII	Pf only	89 (Singer *et al.*, 2004)	76 (Singer *et al.*, 2004)
				97 (Richardson *et al.*, 2002)	96 (Richardson *et al.*, 2002)
ParaHITf®	Dipstick	HRPII or Pan pLDH	Pf only / All species	87.5 (Singh *et al.*, 2005b)	97 (Singh *et al.*, 2005b)
				66.7 (Belizario *et al.*, 2005)	95 (Belizario *et al.*, 2005)
				97 (Singh *et al.*, 2005a)	95 (Singh *et al.*, 2005a)

2.2.2.1 Test Performance of the fluorescent microscopy

2.2.2.1.1 Quantitative Buffy Coat (QBC) method

The QBC method uses Acridine Orange (AO) as the fluorochrome to stain the nucleic acids of any malarial parasites in the sample (Figure 5). Although AO is a very intense fluorescent stain, it is non-specific and stains nucleic acids from all cell types (Moody, 2002). AO is considered hazardous and needs special disposal requirements, making it inappropriate for use in the field. Comparing methodologies, the QBC is more demanding technically (Agabani *et al.*, 1994) and require special equipment and supplies making it more expensive (Craig *et al.*, 1997). However, QBC is rapid and has a high *P. vivax* detection rate (sensitivity and specificity: 87.2% and 95% respectively) (Wang *et al.*, 1996) but lower *P. falciparum* detection rate (sensitivity and specificity: 55.9 % and 88.8% respectively) (Adeoye *et al.*, 2007).
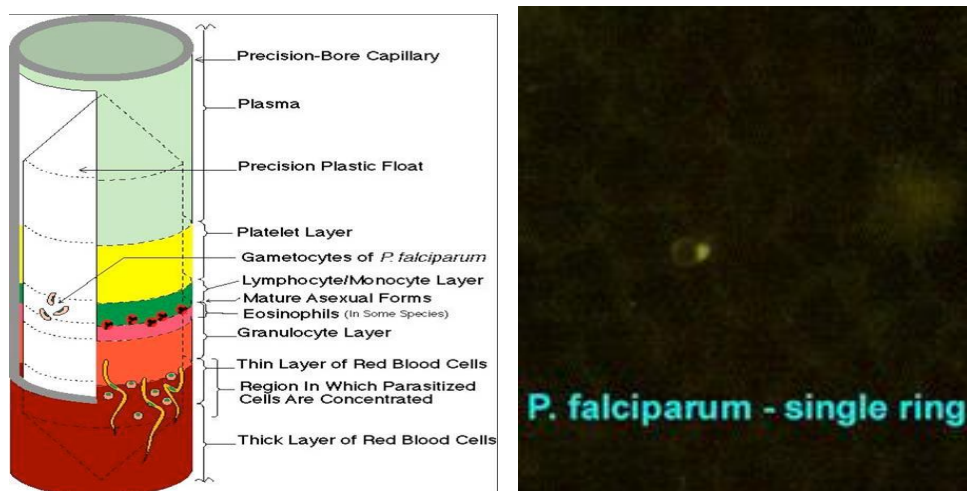
Figure 5: QBC Test showing discrete bands and a parasite

Source: http://www.malariasite.com/MALARIA/QBC.htm

2.2.2.1.2 KAO and BCP method

Whilst the Kawamoto method uses AO as the fluorochrome to stain the nucleic acids of any malarial parasites in the sample, the BCP is used for the BCP method. Both methods are rapid even though the KAO is simpler (Kawamoto, 1991a; Kawamoto, 1991b). The BCP can be applied directly to a lysed blood suspension or to an unfixed but dry thick blood film and stains the nucleic acid of viable *P. falciparum* parasites intensely (Figure 6) and has a reported sensitivity and specificity of >95% for *P. falciparum* (Moody, 2002). The sensitivity of AO staining with parasite levels of <100 parasites/ ml has been reported to range from 41.7%- 93% (Lowe *et al.*, 1996) and specificity of AO staining for *P. vivax* infections appears to be about 52%, whereas that for *P. falciparum* infections is around 93% (Clendennen, 1995). Both methods cannot distinguish between the various plasmodium species.
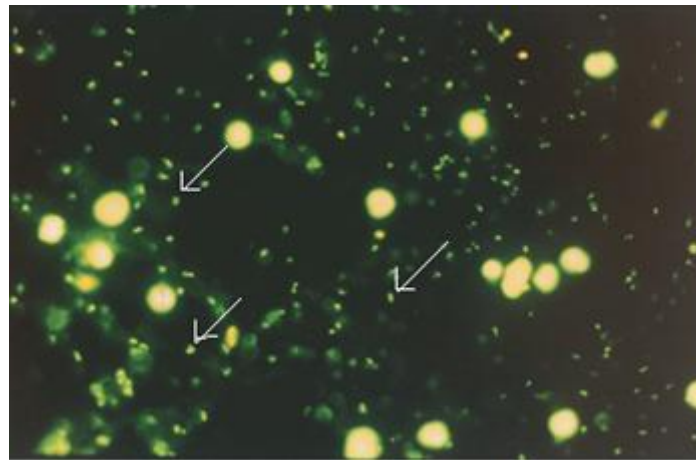
Figure 6: Trophozoites of *P. falciparum* (arrowed) stained with BCP in the fluorescence method.

Source: Moody, 2002

2.2.2.1.3 Partec Rapid Malaria Test®

The test is done using the Partec CyScope®. It is a new innovative microscope that uses both UV fluorescence light and transmitted light simultaneously or in separate and integrates the most recently available generation of powerful light emitting

diode(LED) light sources (Figure 7). It is battery-operated and mobile, designed for several hours of use completely independent from any regular power supply. The Partec CyScope® is perfectly suited for all applications in light and fluorescence microscopy (Nkrumah *et al.*, 2010) and has ready-prepared and ready-to-use test slides which carry the dried-in reagents (DAPI), (emission 443 nm, wavelength 365 nm, safely on the slide surface). Therefore long-term storage and shipment are significantly supported, making malaria testing easier, faster and more affordable than ever before (Nkrumah *et al.*, 2010). Like the other fluorescent methods, this method may not be ideal for species identification.

10 µm
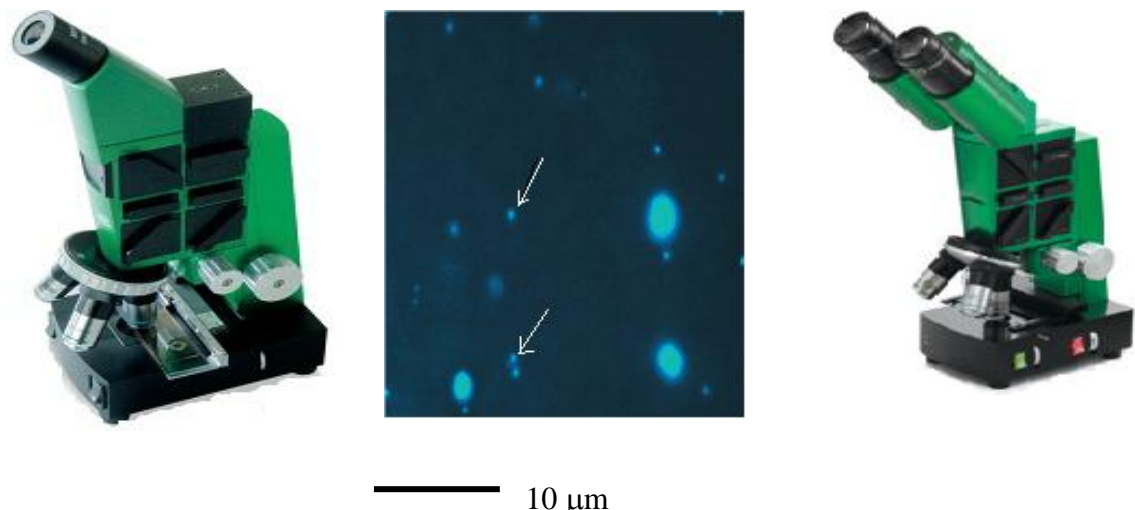
Figure 7: Fluorescent nuclei of *Plasmodium* parasites (arrowed) within unstained peripheral erythrocytes under the Partec CyScope. The large fluorescent round areas represent the nuclei of leukocytes. LED fluorescence light (365 nm), 1000-fold magnification.
Source: www.partec.com

2.2.3 Molecular Techniques

Molecular techniques such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence-Based Amplification (NASBA) have been recently developed in the molecular diagnosis of malaria. Since 1990, several experimental assays have been reported that use various primers, extraction and detection techniques (Snounou *et al.*, 1993b). Several reports have shown that PCR has a higher sensitivity (infection with

five parasites or less per μl can be detected with 100% sensitivity and specificity) (Kawamoto *et al.*, 1996) than examination of thin blood smears, especially in cases with low parasitaemia or mixed infection (Makler *et al.*, 1998). PCR is said to have a lower detection limit of between 0.7 and 0.02 parasites/μl (Schneider *et al.*, 2005). Quantitative-nucleic acid sequence-based amplification (QT-NASBA) can detect parasites at a level as low as 0.02 parasites/μl blood and allows for precise quantification of the parasite load over a range of 20–108 parasites/ml blood (Schoone *et al.*, 2000). However, most published PCR assays are gel based with (Brown *et al.*, 1992)or without (Alves *et al.*, 2002) subsequent probe hybridizations, resulting in a lengthy procedure not optimal for clinical use. The need for a more sensitive and time-efficient assay has led to the development of molecular assays involving Real time PCR (Lee *et al.*, 2002). Real time PCR assays have the potential to detect low levels of parasitaemia, identify mixed infections, and allow for precise differentiation of species via melting curve analysis (Mangold *et al.*, 2005). In a study conducted by Mangold et al., (2005), patient specimens infected at 0.01 to 0.02% parasitaemia densities were detected by Real time PCR, and analytical sensitivity was estimated to be 0.2 genome equivalent per reaction (Mangold *et al.*, 2005).

2.2.3.1 Test Performance of Real time PCR

 Real time PCR is much easier to perform because it offers the option of using single probes instead of multiple probes with complex procedures. It is less time consuming since results interpretation is not gel based but rather on melting curve analysis which takes less time with 100% sensitivity and specificity (Boonma *et al.*, 2007). Real time PCR also prevents carryover contamination as products are not reopened for gel based electrophoresis. On the other hand, the thermal cyclers, primers, probes etc used for the amplification processes are very expensive and therefore cannot be used for

district hospitals and malaria endemic areas where they are needed most. It also requires much expertise and experience which are not available in these endemic areas (Brown *et al.*, 1992; Mangold *et al.*, 2005).

2.2.4 Flow Cytometry

Flow cytometry carries some potential as an alternative tool for malaria diagnosis. Whereas this method appears to be too expensive for malaria-endemic countries, it might be of great value in affluent countries where flow cytometric blood cell differentiation is used routinely in hematology laboratories. An advantage of the method is its potential to detect cases in the absence of clinical suspicion (Hänscheid *et al.*, 1999; Hänscheid *et al.*, 2000). Recent studies using automated hematology analyzers have demonstrated unexpected abnormalities in differential white blood cell plots and reticulocyte histograms from patients with malaria. Normal monocytes can be discriminated from monocytes that have ingested the malarial breakdown product hemozoin because of the ability of hemozoin to depolarize laser light used for routine differentiation of eosinophils. Nuclear material of intraerythrocytic malaria parasites could be discriminated by fluorescent nucleic acid dye used in routine quantification of reticulocytes. The presence of infected erythrocytes leads to a distinct fluorescent spike in reticulocyte histograms, referred to as pseudoreticulocytosis. It has been suggested that this novel method is a useful addition to conventional microscopy (Hanscheid *et al.*, 2001; Hoffmann *et al.*, 1999; Mendelow *et al.*, 1999). Recently, depolarized light scatter of white blood cells has been applied to automated malaria diagnosis using commercial hematology analyzers such as the Cell-Dyn® 3500 (CD3500) (Abbott, Santa Clara, CA) (Mendelow *et al.*, 1999). This allows malaria diagnosis by detecting malaria pigments in white blood cells during routine full blood

counts. Compared to microscopy its sensitivity and specificity is 95% and 88% respectively (Hanscheid *et al.*, 2001).

2.2.5 Mass Spectrometry

A novel method for the in vitro detection of the malaria parasite at a sensitivity of 10 parasites/µl of blood has been recently reported. It comprises a protocol for clean-up of whole blood samples, followed by direct ultraviolet laser desorption time-of-flight mass spectrometry. Intense ion signals are observed from intact ferriprotoporphyrin IX (heme), sequestered by malaria parasites during their growth in human red blood cells. The heme group is photoactive and turns out to be easily detectable by direct laser-desorption mass spectrometry. The laser-desorption mass spectrum of the heme is structure-specific, and the signal intensities are correlated with the sample parasitaemia. Many samples could be prepared in parallel and measurement per sample may not take longer than a second. However, even though this technique may be fast, it is expensive thus cannot be used in developing countries let alone rural areas (Demirev *et al.*, 2002; Mann, 2002).

2.2.6 Malaria Magnetic Deposition Microscopy (MDM)

In an attempt to overcome some problems inherent to blood smear microscopy, a magnet-based approach to concentrate malaria parasites and augment detection of malaria-infected erythrocytes by microscopy has been developed. This system, malaria magnetic deposition microscopy (MDM), exploits the fact that *Plasmodium* parasites produce a crystalline by-product, hemozoin, from heme which is liberated during hemoglobin digestion (Nalbandian *et al.*, 1995; Paul *et al.*, 1981). MDM captures parasitized erythrocytes in a narrow magnetic field and deposits them directly onto a small region of a polyester slide, which is immediately ready for fixation and staining. By concentrating parasites, MDM increases the sensitivity of

diagnosis and decreases the time it takes to read the slide and it the ability to concentrate parasites of all four human malaria parasite species, including efficient capture of *P. falciparum* gametocytes. P. falciparum–infected blood samples were enriched 40-fold from a parasitaemia of 2.7% to nearly 100% whilst P. vivax–infected blood samples were enriched up to 250-fold, from an initial parasitaemia of 0.1% to clusters with 25% infected erythrocytes (Zimmerman *et al.*, 2006).

2.2.7 Light microscopy

The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wrights, or Fields stain (Warhurst *et al.*, 1996).

2.2.7.1 Test Performance

2.2.7.1.1 Giemsa stain

Giemsa microscopy is still regarded as the gold standard and the most suitable diagnostic instrument for malaria control because it is inexpensive to perform, able to differentiate malaria species and quantify parasites (Jonkman *et al.*, 1995). However, microscopy is labour-intensive, time-consuming, requires well-trained (Reyburn *et al.*, 2004), competent microscopists and rigorous maintenance of functional infrastructures plus effective quality control (QC) and quality assurance (QA) (Wongsrichanalai *et al.*, 2007). Microscopic diagnosis has been shown to be insensitive and nonspecific, especially when parasitaemia are low or mixed infections are present (Amexo *et al.*, 2004) . Sensitivities and specificities as low as 71–72% have been reported (Arai *et al.*, 1996; Snounou *et al.*, 1993a).

2.2.7.1.2 Fields stain

Field's stain is widely used as a rapid staining technique for thick and thin blood films for the diagnosis of malaria. This is mostly due to the fact that this technique is easy, quick and the stains are commercially prepared, ready for use and malaria films stained by this method show adequate staining of all stages of Plasmodium including the Schueffner's and James's dots of *P. vivax* and *P. ovale* respectively. However, just as Giemsa stain, a considerable amount of expertise is needed to identify malaria parasites and the stain also fades with time. Compared to Giemsa, the sensitivity of Field's stain is low; 34.57% even though it has an excellent specificity, 100% (Mendiratta *et al.*, 2006; Moody *et al.*, 1985) but other studies have reported a higher sensitivity of 96.3% and a specificity of 96.3% (Ibrahim, 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

A hospital-based longitudinal study was conducted at the Agogo Presbyterian Hospital in the Asante Akim North District (AAND), Ashanti Region. Children up to 16 years attending the Child Welfare (U5) Clinic of the hospital from June, 2007 to May, 2008 were recruited after fulfilling the inclusion criteria.

3.1.1 Study Area

The Asante Akim North District is one of the 21 Districts in the Ashanti Region. The District is located in the eastern part of Ashanti Region. It covers a land area of 1,160 sq. km with an estimated population of about 143,000 (projection from 2000 Population Census). Over 40% of the population is under 15 years of age; Over 50% is under 20. Population aged 65 and above consists of 6.4% of the total population. The vegetation of the study area is mainly rain forest. The climate is tropical. The temperature variation is between 20 and 36 with monthly rainfall varying from 2.0mm in February to 400mm in July. The major occupation of the people is subsistence farming, Animal Husbandry and Forestry. The sub-districts are Konongo- Odumasi (District Capital), Agogo, Juansa, Dwease-Praaso and Amanteman (Figure 8). Most prevalent diseases in the district are malaria, diarrhoea, buruli ulcer and typhoid. The major sources of water in the district include pipe borne, borehole, stream, well and others. Environmental Health and Sanitation issues are major problems facing the district. The malaria endemicity rate for the catchment area was 42.5% in 2007 and 26.5% in 2008 (Unpublished data, Biostatistics Dept. Agogo Presbyterian Hospital).

MAP OF ASANTI AKIM NORTH DIST. OF ASHANTI REGION, GHANA
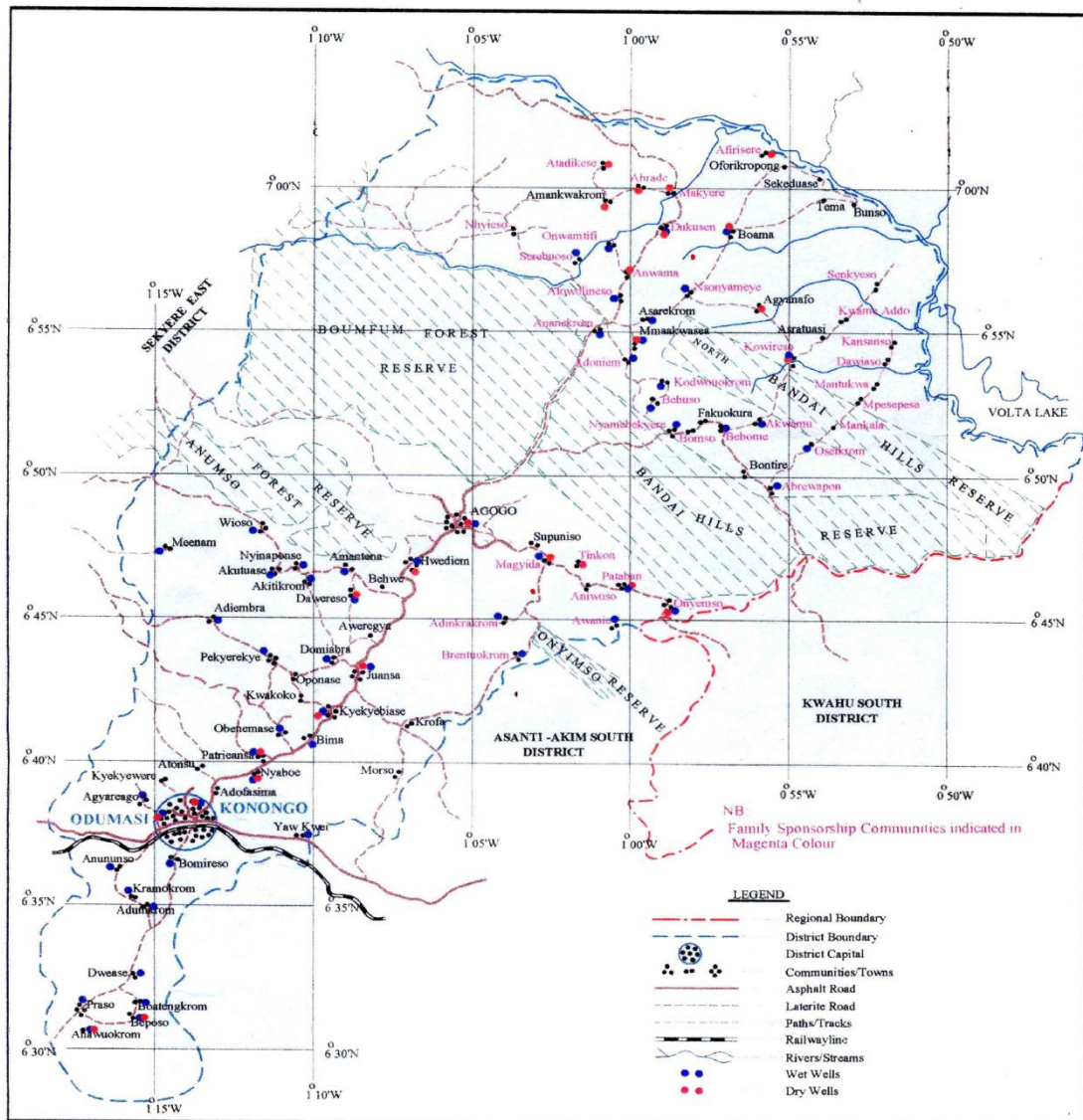
Figure 8: A Map showing the Asante Akim North District of the Ashanti Region
Source: Ghana Surveys Department

3.1.2 Study Site

The Agogo Presbyterian Hospital is a major hospital serving the Asante-Akim North

District and other parts of the Ashanti region. The main departments of the hospital

are the Children's, Casualty, Surgical, Medical, Obstetrics and Gynecology and the

laboratory Departments. The laboratory department of the hospital offers diagnostic

as well as research services. The department is fully equipped with two modern

automated blood culture incubators (BACTEC$^®$ 9050), a carbon dioxide incubator, two safety cabinets for bacteriological culture and sensitivity testing, two Sysmex KX21N and one Sysmex XS1000i Haematology autoanalysers, one Vitalab Selectra Junior autoanalyser and two Vitalab Flexor E autoanalyser for Biochemistry. There are 6 Light microscopes, 5 scientific fridges and 2 freezers (-20°C and -80°C) as well as a refrigerated centrifuge and a water bath. Laboratory tests are carried out from approved Standard Operating Procedures (SOPs) and every activity undertaken in the laboratory is well documented. Personnel of the laboratory participate in various External Quality Assessment programmes such as the United Kingdom External Quality Assessment Scheme (UK NEQAS) for Haematology and Royal College of Pathology Australia (RCPA) for Clinical Chemistry and the National Institute of Communicable Diseases/ National Health Laboratory (NICD/NHL), South Africa and the Malaria Diagnostic Centre for Excellence (MDCoE), Kisumu-Kenya in Microbiology (Bacteriology) and Malaria Parasitology (microscopy) respectively.

3.1.3 Study Population

Children attending the U5 Clinic of the hospital who fell within the enrollment criteria were included in this study.

3.1.3.1 Inclusion criteria

Children attending the U5 clinic for the first time with fever or a history suggestive of malaria and had not taken any antimalaria drugs within fourteen days of reporting to the hospital were included in this study. Signed informed consent was obtained if the potential parent demonstrated understanding of the study and was willing to enroll. In the case of an illiterate parent, a left thumbprint was obtained on the consent forms and a separate witness consent form was signed by a literate witness who had

observed the consent processes. The interview was done in Twi which is the local language in the district.

3.1.3.2 Exclusion criteria

Children who were attending the U5 clinic for reviews and those who had taken any antimalaria drugs within fourteen days of reporting to the hospital were not included in this study.

3.2 Sampling

Patients were recruited randomly. This consisted of the first ten eligible patients for each day. The laboratory request slip indicating the requested test was placed at the sample collection area after which patient details were entered into a sample collection book and assigned a laboratory number. The request form and an EDTA tube were labelled with the patient's laboratory number. Patients were made to sit comfortably. For babies and toddlers, they were assisted by their mothers or guardians and the procedure was explained. Using methylated spirit the middle finger or heel (in the case of babies) was disinfected. A sterile lancet was used to prick the cleaned area of the middle finger or heel deep to allow free flow of blood and disposed into a safety puncture/sharps container. A maximum volume of 1.5ml blood was collected into the sterile container and mixed well by turning up and down about six times to prevent the blood from clotting. The pricked site was cleaned with a new swab and plastered where necessary. Blood samples and request forms were transferred to the laboratory as soon as possible for processing.

3.2.1 Slide Preparation, Staining and Reading

See Appendix 4.

3.3 Laboratory investigation

3.3.1 Sample Processing

Blood samples with accompanying request forms were transported immediately to the laboratory for processing. Three immediate tests: Partec rapid malaria test (PM), Binax Now rapid diagnostic test (BN RDT) and Giemsa stain (GS) and one later test: Real time PCR was done. The three immediate tests were done by well trained laboratory staff independently and blinded to each other's results. Plasmodial DNA was extracted from the remaining samples and stored at -20°C. These were later transported to the Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg-Germany for amplification. Smear preparation of the blood on clean dry 76mm x 26mm microscopy slide was stained with the Giemsa stain (See Appendix 4).

3.3.2 Giemsa stained Microscopy

The Giemsa stained films were examined with a Zeiss light microscope (Aziostar plus, Carl Zeiss Microimaging, Germany) using the high power (40x) and the oil immersion (100x) objectives (Figure 9).
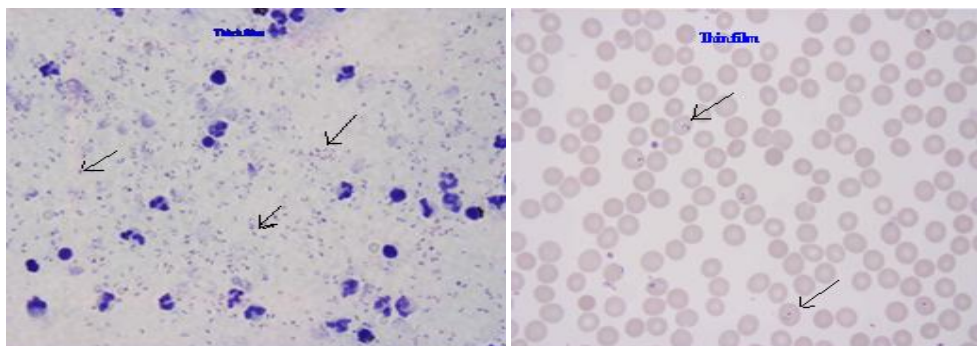
Figure 9: Giemsa stained malaria parasites (arrowed) as they appear in the thick and thin films under the light microscope

Source: MDCoE, Kisumu, Kenya

3.3.3 Partec rapid malaria test®

The test was done according to the manufacturer's instructions (See Appendix 2). Parasite examination was done with the PM using the oil immersion (100x) objectives. The presence of small bright spots (Figure 10) indicated the presence of malaria parasites.
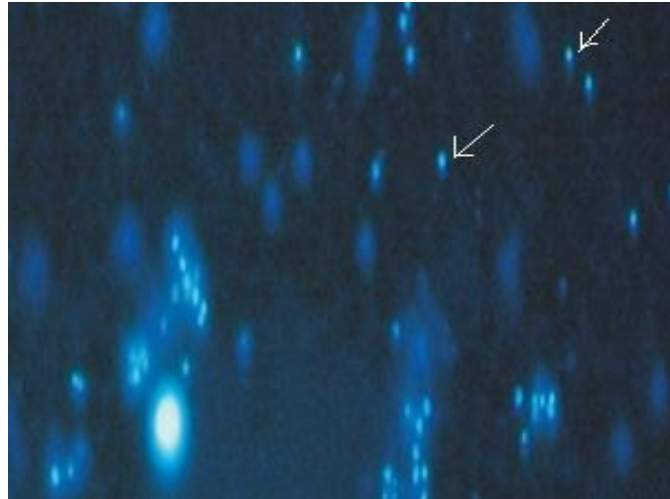
Figure 10: DAPI stained malaria parasites as they appear under the fluorescent microscope (arrowed)

Source: www.partec.com

3.3.4 Binax Now® RDT

The test and result interpretation were done according to the manufacturer's instructions (See Appendix 1).

3.3.5 DNA extraction

The QIAGEN® FlexiGene DNA kit was used in this study. Reagents were prepared and extraction done following the manufacturer's instructions (See Appendix 3).

3.3.6 DNA amplification

Plasmodium detection was performed using Real time PCR in the Corbett Rotor-Gene 6000® Light Cycler (Corbett Life Science, GmbH). The 18s rRNA gene was chosen

as the target since it contains both highly conserved and variable regions, and at least five copies of the gene are dispersed on separate chromosomes of the *Plasmodium* genome (Gardner *et al.*, 2002; McCutchan *et al.*, 1995). A universal primer was used. This could detect all the four species of Plasmodium. The primer sequence was: (PL 1473 F) 5′-TAA CGA ACG AGA TCT TAA- 3′ and (PL 1679 R) 5′- GTT CCT CTA AGA AGC TTT- 3′ (Mangold *et al.*, 2005). For one single reaction, the reaction mix contained 2 μl Plasmodia DNA, 2 μl SYBR Green mix, 1.0 μl PL 1473 F 18, 1.0 μl PL 1679 R 18 and 4.4 μl MgCl$_2$ with a concentration of 25 mM and 9.6μl PCR grade water making a total amount of 20 μl. Melting curve analysis was used to determine the final product. The melting temperatures for the *Plasmodium spp* were as follows: *P. malariae*- 73.5-75.5 °C, *P. falciparum*- 75.5-77.5 °C, *P. ovale*- 77.5-79.0 °C and *P. vivax*- 79.0-81.0 °C

All primers were obtained from MWG diagnostics Inc., (Netherland) and kept at 4°C. Reaction conditions were chosen according to a standard Rotor Gene protocol at the BNITM Molecular Parasitology laboratory and were 10 min at 95°C (Pre-incubation), 40 cycles of 10sec at 95°C (Denaturation), 5sec at 60°C with touchdown at 0.5 °C for 11 cycles (Annealing) and 20sec at 72°C (Extension), then 2min at 95°C and 30sec at 68°C (Denaturation and cooling), 2min at 95°C , 30sec at 60°C and 5sec at 68°C (Starts at 68°C, increases at each step by 0.6°C, holds for 5 seconds and measure fluorescence at 470-510nm) (melting) and a cooling step at 40°C for 20sec.
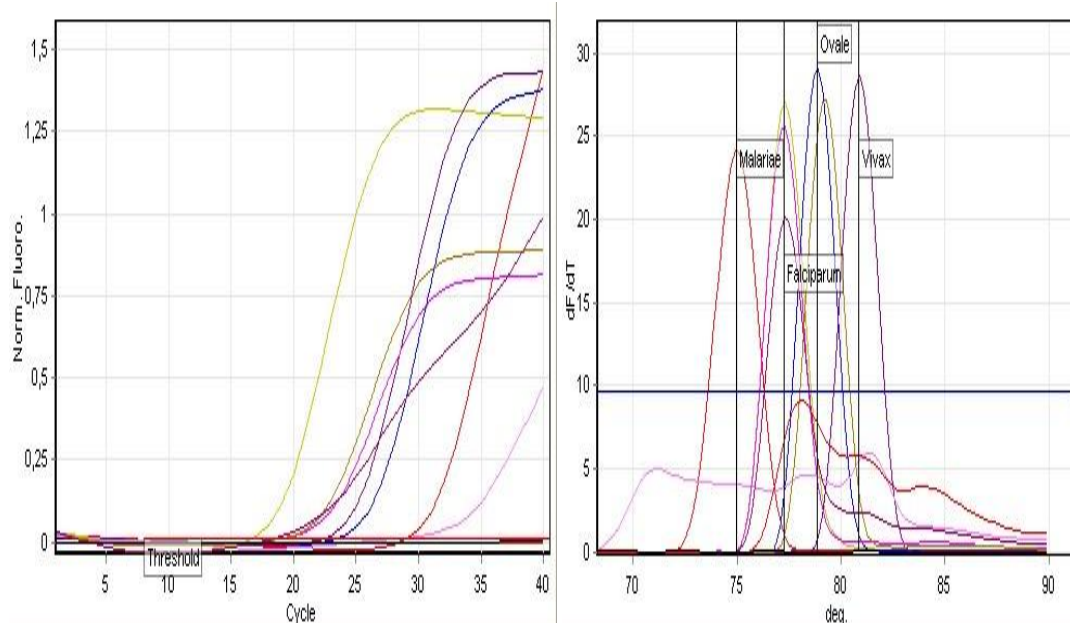
Figure 11: Quantitation data and melting curve reports used for result interpretation
Source: Original

3.4 Data analysis.

All data were double-entered into a predesigned electronic database using Epi info version 6.04dfr (Center for Disease Control, Atlanta, GA, USA) and cleaned regularly. Data was exported to *Stata/*SE9.0 statistical software (Stata Corporation, Texas USA) for analysis. Sensitivities, specificities, positive predictive, negative predictive values and the kappa statistics were determined for the various tests and compared with one another. A p value less than 0.05 was considered significant in all comparison.

3.5 Reference Standard

An expanded reference standard (Thejls *et al.*, 1994) comprising all test results that were concordant for the test methods was used as the reference standard for the diagnostic comparison.

3.6 Ethical Approval

Ethical approval for the study was obtained from the Committee on Human Research, Publication and Ethics (CHRPE), of the School of Medical Sciences, KNUST-Kumasi. This study was part of a major study on Neglected Infectious Diseases in the District.

CHAPTER FOUR

RESULTS

A total of seven hundred and fifty one (751) participants were involved in this study. Out of this 400(53.3%) were males and 351(46.7%) were females. Participants' age ranged from 3 to 16 years with the modal age being 4 years (150/751) (Figure 12) and the mean age being 9.5 years. Whilst 638 (85.0%) of the study participants were Akans, 76 (10.0%) were Northerners, 19 (2.5%) were Ewes, 6 (0.8%) were Gas and 13 (1.7%) were from other ethnic groups. 450(59.8%) of the study population were from the Agogo township whilst 67 (8.9%) were from the Konongo township (Figure 13).

Figure 12: Age Distribution of all study participants

Figure 13: Distribution of the place of residence of study participants
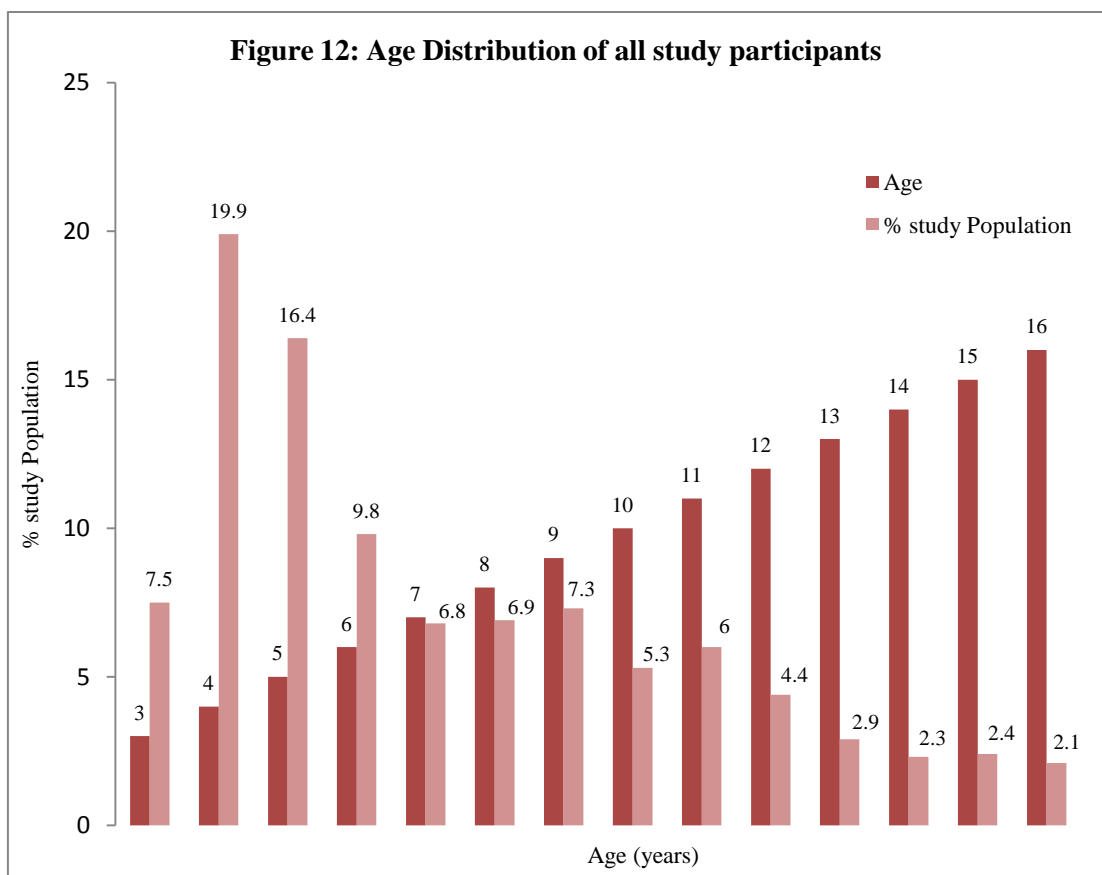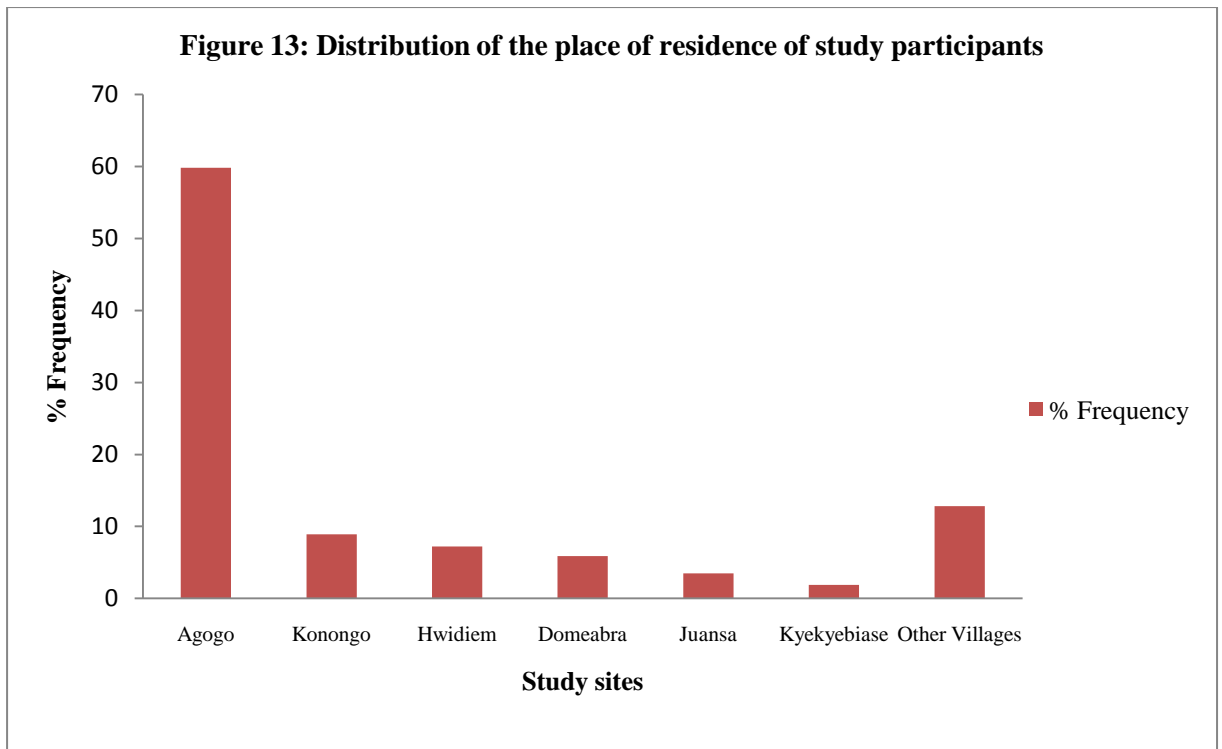
Giemsa stain test and Partec rapid malaria® test were done for all 751 study participants. Whilst Real time PCR was done for 488 (65.0%) participants; Binax Now® RDT (BN RDT) was done for 263 (35.0%) participants. 289/751 (38.5%), 298/751 (39.7%), 288/489 (58.9%) and 114 (43.3%) were positive for Giemsa stain (GS), Partec rapid malaria® test (PM), Real time PCR and Binax Now® RDT respectively as shown in table 2.

Table 2: The total number of positives and negatives for each test in the study

Test	Positive (%)	Negative (%)	Total
GS	289 (38.5%)	462 (61.5%)	751
PM	298 (39.7%)	453 (60.3%)	751
Real time PCR	288 (59.0%)	200 (41.0%)	488
BN RDT	114 (43.3%)	149 (56.7%)	263

There were 13 discordant results between GS and PM (Table 3). Of these GS detected 2 positives which were negative for PM. The two were also positive for Real time PCR (Ct = 27.6). PM detected 11 positives which were negative for GS with three being positive for Real time PCR (Ct = 28.6). There were also 117 discordant results between PM and Real time PCR of which 109 were positive for Real time PCR but not PM and 17 discordant results between PM and BN RDT with 10 being positive for BN RDT but not PM. BN RDT gave negative results for one *P. malariae* and one *P. ovale* infection even though all the other test methods gave positive results. Using an expanded reference standard the sensitivities of GS, PM, Real time PCR and BN RDT was 96.7% (95% CI: 93.9-98.4), 97.3% (95% CI: 94.8-98.8), 97.3% (95% CI: 93.8-99.1) and 96.5% (95% CI: 91.2-99.0) respectively. The specificities were 100% (95% CI: 99.2-100), 98.5% (95% CI: 96.8-99.4), 64.6% (95% CI: 58.9-70.0) and 96.7% (95% CI: 92.4-98.9) respectively. The Positive Predictive Value (PPV) for GS, PM, Real time PCR and BN RDT was 100% (95% CI: 98.7-100), 97.7% (95% CI: 95.2-99.1), 62.8% (95% CI: 57.0-68.4) and 95.6% (95% CI: 90.19-98.6) respectively whilst the Negative Predictive Value (NPV) was 97.8% (95% CI: 96.1-99.0) , 98.2% (95% CI: 96.6-99.2) , 97.5% (95% CI: 94.3-99.2) and 97.3% (95% CI: 93.3-99.3) respectively (Table 4).

Table 3: A summary of all test results in the study

		GS		PM		Real time PCR		BN RDT	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
GS	Pos	289	-	-	-	178	4	-	-
	Neg	-	462	-	-	110	196	-	-
PM	Pos	287	11	298	-	-	-	104	7
	Neg	2	451	-	453	-	-	10	142
Real time PCR	Pos	-	-	179	109	288	-	-	-
	Neg	-	-	-	192	-	200	-	-
BN RDT	Pos	104	-	-		-	-	114	-
	Neg	3	146			-	-	-	149

Table 4: Performance characteristics of the test methods using the expanded reference standard

Test Methods	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
GS	96.7 (93.9-98.4)	100 (99.2-100)	100 (98.7-100)	97.8 (96.1-99)
PM	97.3 (94.8-98.8)	98.5 (96.8-99.4)	97.7 (95.2-99.1)	98.2 (96.6-99.2)
Real time PCR	97.3 (93.8-99.1)	64.6 (58.9-70.0)	62.8 (57.0-68.4)	97.5 (94.3-99.2)
BN RDT	96.5 (91.2-99.0)	96.7 (92.4-98.9)	95.6 (90.1-98.6)	97.3 (93.3-99.3)

There was a strong agreement between GS, PM, BN RDT and the reference standard: k=0.97, 0.96 and 0.93 respectively but a poor agreement when compared with Real time PCR (k=0.56) (Table 5). Compared to each other, the tests methods had a strong agreement as well: GS vs. PM, k= 0.96 and for PM vs. BN RDT, k=0.87, see table 6. Real time PCR had a higher positive detection rate then the other methods; 110/488 (22.5%) and 109/488 (22.3%) of the total samples were positive for Real time PCR but not GS and PM respectively.

Table 5: A table showing the expected and observed agreements of the various tests with the reference standard and their corresponding P and K values

Test Methods	Expected Agreement (%)	Observed Agreement (%)	Standard Error	P Value	K Value
GS	52.4	98.7	0.04	< 0.001	0.97
PM	52.1	98	0.04	< 0.001	0.96
Real time PCR	47.9	77.1	0.04	< 0.001	0.56
BN RDT	50.9	96.6	0.06	< 0.001	0.93

Table 6: A table showing the level of agreement between all test methods

	GS vs. PM	GS vs. Real time PCR	GS vs. BN RDT	PM vs. Real time PCR	PM vs. BN RDT
k value	0.96	0.55	0.9	0.54	0.87
Standard error	0.04	0.04	0.06	0.04	0.06
p value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Parasite counts obtained from the PM (mean count: 462.6 parasites/µl; 95% CI: 393.6-531.6; STD: 597.7) were relatively lower than that obtained from the GS (mean count: 737 parasites/µl; 95% CI: 596.3-877.8; STD: 1219.7) with p value < 0.001.

Figure 14: Distribution of log parasite count using Giemsa stain and Partec rapid malaria test
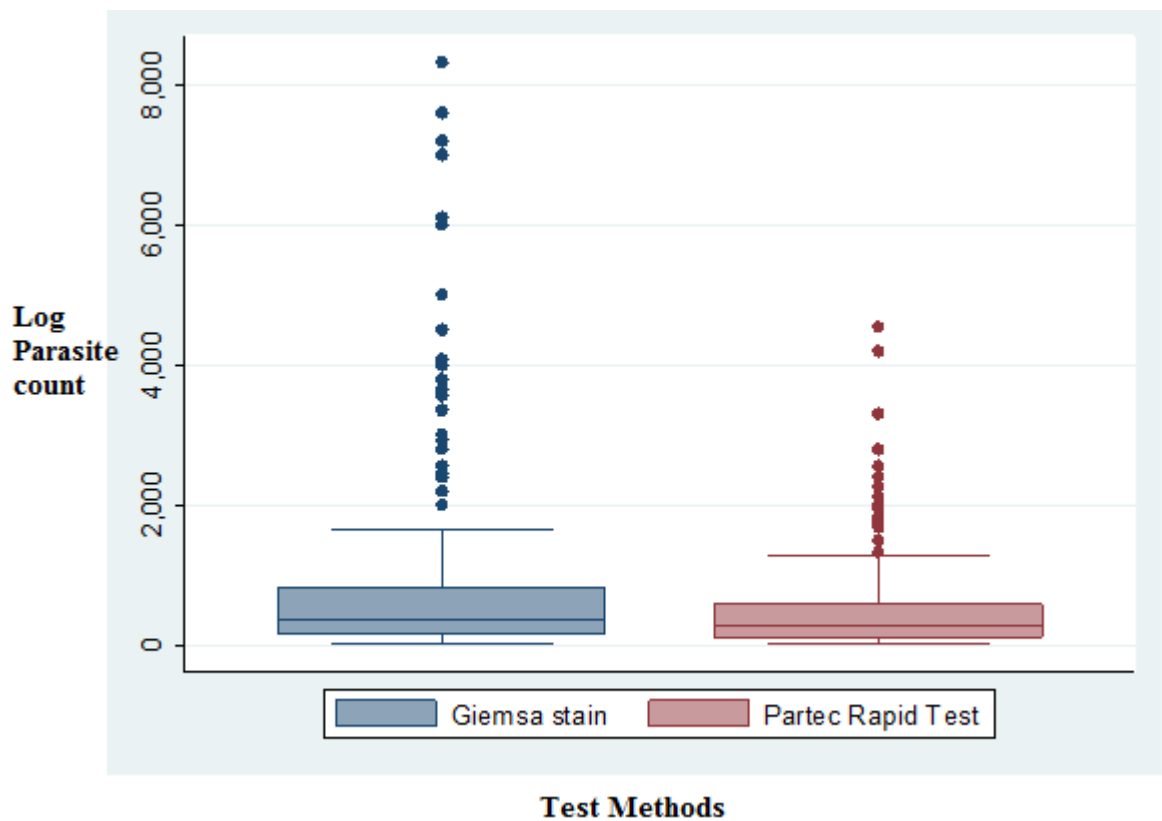
Mean and interquartile ranges of malaria parasite counts in the peripheral blood of children as detected by Giemsa stain and Partec Rapid Test® technique.

Less time (5 minutes) and training was involved in the use of the PM than the GS. The operational characteristics of the tests were also compared using several variables. Whilst the PM test had the shortest and simplest steps involved in the testing process, Real time PCR had the longest and most complex. From the manufacturer's the average cost of a PM microscope was lesser than that of a GS microscope and the BN RDT required the smallest quantity of blood for testing (Table 7).

Table 7: Comparison of the operational characteristics of PM to the other test methods

Parameters	Giemsa stain	Partec Rapid test [®]	Real time PCR	BN RDT
Steps involved in performing test	6 steps	3 steps	> 20 steps	6 steps
Average time/test	25min*	5 min**	270min*	15min**
Average cost/test	$1.00[§]	$0.50 **	$8.80[§]	$1.00**
Average cost/microscope	$3,000[¥]	$2,500**	$31,031[§]	NA
Blood quantity/test	$10\mu l^{Z}$	$5\mu l$**	$20\text{-}100\mu l$**	$1\mu l$**
Electric current by a standby battery	No	Yes**	No	NA

* Time observation made in this study
** Data obtained from the manufacturer's and test procedures.
[§]Figure obtained from Study site
[¥] Microscope cost from manufacturer
[Z] Blood quantity used in this study
§ Boonma et al., 2007

CHAPTER FIVE

DISCUSSION

In malaria-endemic areas of Ghana, the predominant *Plasmodium* species is *P. falciparum* thus making it an important cause of severe malaria (UNDP, 2002). The disease accounts for an average of 13.2% of all mortality cases in Ghana and 22% of all mortalities in children under 5 years. In the case of pregnant women, out of the total number reporting at the health institutions, 13.8% suffer from malaria and 9.4% of all deaths in pregnant women (Antwi *et al.*, 1998). It is estimated that malaria prevalence (notified cases) is 15,344 per 100000 with a malaria death rate for all ages being 70 per 100 000. In the case of the 0 − 4 years, it is 448 per 100 000 reported for the year 2000 (UN, 2003). Early treatment is therefore a key measure in the management of severe malaria. For this to be accurate, early and definitive diagnosis must be available (Bojang *et al.*, 2000). Giemsa stained microscopy has historically been the mainstay of malaria diagnosis and continues to be the reference standard despite its disadvantages (Gilles, 1993). As a result of these limitations, alternative techniques that are easy and quick to perform as well as cheaper for the diagnosis of malaria need to be evaluated.

The present study shows that the PM has a better diagnostic ability than the GS, making it a good alternative to the Giemsa thick film. This correlates with the findings of a study conducted in Sudan (El-Din *et al.*, 2010) and Ghana (Nkrumah *et al.*, 2010). The PM detected more positive malaria cases when compared to the GS. This could be attributed to the fact that the PM uses a non specific fluorescent dye- 4'-6-Diamidino-2-phenylindole (DAPI) - which detects intracellular double stranded DNA that may be present in erythrocytes; non plasmodial DNA might have been misinterpreted as plasmodial DNA. On the other hand, the three false negative results could have been caused either by the low level of parasitaemia in blood films as also reported by Milne and his team (Milne *et*

al., 1994) who found that most routine diagnostic laboratories generally achieved a lower sensitivity of detection (average, 0.01% RBC infected, 500 parasites/μl) in an examination of blood films or by semi-immune parasite carriers with low parasitaemia. The three false negative results had an average cycle time of ~ 28.6 when analyzed with Real time PCR which has a higher sensitivity and specificity (100%) (Boonma *et al.*, 2007). This goes to support the hypothesis that, low parasitaemia levels made it virtually impossible for parasite detection through microscopy.

The BN RDT detects the presence of plasmodial antigens HRP2 and aldolase. The HRP2 antigen has been shown to persist up to 14 days after treatment (Moody, 2002). This might have contributed to the increased number of false positive tests when compared to GS and PM. For this reason also, the BN RDT may not be a good test method for treatment monitoring even though its sensitivity and specificity appears to be good. The BN RDT can detect parasite levels above 20 parasites/μl for *P. falciparum* and ≤100 parasites/μl for *P. vivax* (Wongsrichanalai *et al.*, 2003). False negative results when compared to GS and PM could have been attributed to the fact that parasite antigen levels were too low to be detected as a result of very low parasitaemia during early stages of infection.

In a study conducted by the WHO (WHO, 2008a) to evaluate malaria RDTs to produce performance data, the BN RDT performed very well when tested against 20 cultured wild type *P. falciparum* species. At parasite densities of 200μl and 2000μl, the detection rates were 80% and 100% respectively. When subjected to temperatures of 35°C, the parasite detection rate was 100% for both parasite densities and at 45°C, the parasite detection rate was 100% for 200μl and 95% for 2000μl (WHO, 2008a). The BN RDT does not require the use of electricity, can be performed anywhere with little training at an affordable cost.

These confirm the use of the BN RDT as a good point-of-care malaria test for resource limited endemic areas where *P. falciparum* is the main cause of malaria.

The World Health Organization Sexually Transmitted Diseases Diagnostics Initiative (SDI) has developed the ASSURED criteria as a benchmark to decide if tests address disease control needs: **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free and **D**eliverable to end-users (Peeling *et al.*, 2006). Even though this criteria was developed as a benchmark for the evaluation of RDTs for sexually transmitted infections such as syphilis, it can also be used as a benchmark for the evaluation of RDTs for other diseases such as malaria.

Affordability: The BN RDT and PM cost $1.00 and $0.50 per test respectively (Table 7). In rural endemic areas where majority of the people are poor, these tests are affordable enough to be accessed in the health centers, homes etc.

Sensitivity and specificity: The accepted level of sensitivity for a rapid diagnostic test in the diagnosis of malaria is a sensitivity of 95% (WHO/TDR, 2006). Compared to the expanded reference standard, the sensitivities of BN RDT and PM were 96.5% and 97.3% respectively whilst the specificities were 96.7% and 98.5% respectively. Both methods are sensitive and specific enough to be used as rapid diagnostic tools for the diagnosis of malaria in endemic areas.

User-friendly, rapid and robust: Both methods require little training. While it takes twelve days intensive training for Giemsa microscopy (Ohrt *et al.*, 2007), the Partec rapid malaria test takes about three days. It takes only about 5 minutes to obtain results from the PM and 15 minutes from the BN RDT compared to 25 minutes for the GS. The BN RDT can withstand temperatures up to 45 $^{\circ}$C, making it very ideal for tropical conditions.

Equipment-free and Deliverable to end-users: The BN RDT does not require any equipments but the PM does. Nevertheless both methods are deliverable to the end-user. The PM is battery operated and portable (weight 2.7kg) and can thus be carried for field work and to places where there may be no regular electricity.

There is the need therefore to expand malaria diagnostic services as part of a greater framework of health system strengthening within resource-limited settings. Increasingly, countries and implementing partners have identified that limited diagnostic capacity represents a major barrier to implementation and sustainability of prevention, treatment and care programs for malaria (Maputo.Declaration, 2008). The BN RDT and PM therefore present as very good tools in the prevention, treatment and care programs for malaria.

Giemsa stain has been shown to be a less sensitive test to be used as a reference method (Coleman *et al.*, 2006); nevertheless, it is cheap and can be used for both qualitative and quantitative purposes as well as the only cheap method that can be used for species identification (Bejon *et al.*, 2006). The performance of the Real time PCR appeared to be poor when compared to GS and PM, but the data collected indicates that the discrepancy between the GS and PM resulted from the poor performance of GS and PM at low parasite densities. This is in agreement with the findings reported by Jonkman and his team (Jonkman *et al.*, 1995). These data are not unusual when the diagnostic method being evaluated is more sensitive than the reference method. Real time PCR appears to be a useful method for detecting *Plasmodium* parasites in active malaria surveillance (Coleman *et al.*, 2006)due to its high analytical sensitivity (0.01-0.02 parasites/µl) (Mangold *et al.*, 2005). One major drawback of this method is that, plasmodial DNA has been reported to persist for up to 8 days post treatment (Kain *et al.*, 1994) and this can lead to false positive

results. On the other hand, it is not the best tool for rural endemic areas due to its high cost, sophistication, lack of electricity, labour intensiveness and very long turnaround time (Hänscheid *et al.*, 2002).

Averagely, the PM has better diagnostic abilities. Compared to the other test methods, the PM has other advantages; it is faster and less labour intensive and therefore has a better turnaround time and requires very little training and expertise. It is relatively cheaper to use and thus can be afforded by patients in poor endemic areas. It uses rechargeable batteries hence can be used at places where there is no electricity. The battery pack consists of Nickel-Metal Hydride (NiMH) batteries with an in-built temperature probe that ensures optimal recharging process. The lifetime of the batteries however depends on the usage; usually it can be recharged 500-1000 times. The battery pack can be easily exchanged and its readily available (Nkrumah *et al.*, 2010). The charging time is a maximum of 4 hours and a fully charged battery can be used to work continuously for approximately 6 hours. Charging of the batteries can be done while working with the CyScope® (Nkrumah *et al.*, 2010). Little amount of blood is needed thus making it ideal for use in children. Furthermore, there is no need for reagent preparation as they are already dried on the slides ready-to -use. This may also prolong the shelf life of the test reagents. Nevertheless, it has drawbacks too; the test is not suited for species differentiation and it may not be possible to store blood slides for extended periods (Table 7).

Parasite counts obtained from the GS were significantly higher than those obtained from the PM. It is unlikely that parasites are hidden during Giemsa stained microscopy, since adequate preparation of the slide ensures visibility through all planes of focus. The lyses of the red blood cells during the staining process reveals the parasites making them easier to be seen (Moody, 2002). The Partec rapid malaria test employs fluorescence in which red

blood cells are not lysed therefore they may lie on each other or overlap with each other thereby preventing parasites in red blood cells that may be lying beneath other cells from being identified and counted.

5.1 Conclusion

This study has shown that the PM can be used as an alternative method for Giemsa thick film and the BN RDT can be used as point-of-care malaria test device for resource limited areas. More sensitive tests such as the Real time PCR must be used at reference laboratories and institutions to further enhance malaria diagnosis, reduce the use of antimalaria drugs and eventually lead to a reduction in antimalaria drug resistance. For parasite speciation, the Giemsa thin film may be used.

5.2 Recommendations

1. This study has found the PM to be a reliable diagnostic tool that is very sensitive and specific in diagnosing falciparum malaria. Since this is the predominant species in Ghana, and it is the species causing most mortality and complications, this is very relevant and useful. It is expected that the CyScope® will show similar results for other malaria species especially P. *vivax*, but this could not be ascertained by this study. Further studies are needed to determine its effectiveness in diagnosing other Plasmodium species.

2. Based on the ASSURED criteria and the need to expand malaria diagnostic services as part of a greater framework of health system strengthening within resource-limited settings (Maputo.Declaration, 2008), the BN RDT and PM can be considered as a point-of-care diagnostic device for resource limited endemic areas.

5.3 Limitation

In Africa over 70% of malaria cases do not present initially to health facilities but diagnosed and managed at home with traditional remedies or drugs bought from local shops (Amexo *et al.*, 2004). Patients only attend health centers after self-treatment fails (Chandramohan *et al.*, 2002). This might have affected the performance of some of the test methods especially BN RDT.

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APPENDICES

APPENDIX 1

Immunochromatographic Test Principle

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Immunochromatographic tests are based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line. Incorporation of a labeled goat antimouse antibody capture ensures that the system is controlled for migration (Piper *et al.*, 1996). Migration depends on several physical characteristics of the component reagents, primarily the porosity of the membrane controlling the flow rate and the components of the buffer solution used to transport the labeled antigen-antibody complex in the lysed blood sample (Moody, 2002).

Binax Now® RDT Test procedure

- Gently mix the sample by turning it up about six times.
- Fill the capillary tube provided fully and slowly apply the blood to cover the entire purple sample pad on the right side of the device. This is done by holding the capillary tube vertically and gently pressing the end against the purple pad in several places.
- Once the pad is saturated, properly discard the capillary tube.

Note: The test may not require all of the blood that has been collected into the capillary tube.

- If using a pipette; draw 15µl of blood and apply gently to the purple sample pad.

Important: Incorrect addition of sample may lead to an invalid or uninterpretable test.

- Hold the buffer reagent bottle vertically and add two free-falling drops of the buffer to the white pad immediately below the purple sample pad. Allow the first drop to absorb into the pad before adding the second drop.

Note: Do not add the buffer directly to the purple pad.

- Allow the blood sample to run up the full length of the test strip.
- Do not allow the blood to run into or under the absorbent pad at the top of the strip, as doing so will hinder optimal washing (clearance) of the test strip.

Note: If blood flow up the test strip appears to stall or is less than halfway up the strip after one minute, add one additional drop of the buffer to the white pad at the bottom of the test strip (below the sample pad where the blood was added)

- Just before the blood sample reaches the base of the white absorbent pad located at the top of the test strip, slowly add four free-falling drops of the buffer to the wash pad on the top left-hand side of the test device, allowing each drop to absorb into the pad before adding the next.

Note: The third and fourth drops may not be completely absorbed into the pad.

- When the sample just reaches the base of the white absorbent pad at the top of the test strip, remove the adhesive liner from the right edge of the device, and close the device.
- This allows the buffer to wash (clear) the blood sample off the test strip.
- To ensure good device closure and test flow, press very firmly along the entire edge to the right of the result window.
- Read the test result through the viewing window 15 minutes after closing the test device.
- Results read before or after 15 minutes may be inaccurate.

Note: When reading test results, tilt the device to reduce glare on the result window, if necessary.

<div align="center">

Result Interpretation

Valid Test Results

</div>

The Control Line (C) will appear on all valid tests and, when it is present, test results are interpreted as follows. Note that the appearance of any test Line, even when very faint, indicates a positive result.

<div align="center">

Test result description/ Interpretation

</div>

- **T1 Positive:** Positive for *P. falciparum* protein antigen only
- **T2 Positive:** Positive for malaria protein antigen, representing *P. vivax* or *P. malariae* or *P. ovale* or a mix of these. Differentiation of the species is not possible.

- **T1 and T2 Positive:** Positive for *P. falciparum* protein antigen. In some cases this may represent a mix of *P. falciparum* antigen with *P. vivax, P. malariae,* or *P. ovale* protein antigen. Differentiation between a *P. falciparum* only infection and a mixed infection containing *P. falciparum* and another malaria species is not possible with this test.

- **Negative:** Presumptive negative for malaria antigens. Infection due to malaria cannot be ruled out. Malaria antigen in the sample may be below the detection limit of the test. Negative results must be confirmed by thin / thick smear microscopy.

Invalid test results

The test is invalid if the control line (C) does not appear. If this occurs, the test should be repeated using a new test card.

Test Principle for Partec CyScope®

The Partec CyScope® uses DAPI (4', 6-diamidino-2-phenylindole) as the dried-in reagent on the test slide. DAPI is a fluorescent stain that binds strongly to DNA (intraerythrocytic DNA). It is used extensively in fluorescence microscopy. Since DAPI can pass through an intact cell membrane, it may be used to stain both live and fixed cells. For fluorescence microscopy, DAPI is excited with ultraviolet light. When bound to double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm (Du *et al.*, 1998). DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA (Hard *et al.*, 1990) .

Partec CyScope® Test Procedure

- Remove the test slide(s) from the slide box.
- Label them with the corresponding pathology number(s).
- Take a drop of blood from a finger prick directly or from a well mixed blood in an EDTA tube.
- Put the drop of blood onto the Test Slide (delivered ready-prepared, already containing the necessary reagents dried-in for long and safe storage) making sure not to mismatch the samples. The drop of blood must be placed at the portion containing the reagent.
- Cover the slide with a cover glass, wait for a minute and analyze the slide with the Partec CyScope® in a dark room.
- The presence of bright shinny dots (Fig. 11) indicates a positive slide for malaria parasites.
- To prevent the slides from drying out, they must be kept in a wet chamber.

Cell Count

- Parasites were counted against 200 or 500 WBC's.
- For very heavy parasitaemia (>100 parasites/field), an approximate count was done using a quarter of the field.
- The approximate count was then multiplied by four to get the overall total count.

APPENDIX 3

Plasmodia DNA Extraction and Amplification

Reagent content

The QIAGEN® FlexiGene DNA kit consists of the following:

- Lysis buffer (FG1)

- Denaturation buffer (FG2)

- Hydration buffer (FG3)

- QIAGEN® Protease enzyme

Preparation of reagents

QIAGEN® Protease stock solution

- Resuspend the lyophilized QIAGEN® Protease enzyme in the following volumes of FG3 buffer.

- 0.3ml FG3 buffer when using the 50ml FlexiGene DNA kit or

- 1.4ml FG3 buffer when using the 250ml FlexiGene DNA kit.

- Dissolved QIAGEN® Protease enzyme should be stored at 2-8°C or in aliquots at -20°C.

Buffer FG2/QIAGEN® Protease mixture

- Calculate the total volume of blood to be processed.

- For every 1ml blood, mix together 500μl buffer FG2 and 5μl reconstituted QIAGEN® Protease enzyme.

- The buffer FG2/QIAGEN® Protease enzyme mixture should be prepared not more than one hour before use.

Procedure

- Pipette 250μl buffer FG1 into a pre-labeled 1.5 ml centrifuge tube.

- Add 100 μl whole blood, mix by inverting the tube five times.

- Centrifuge for 20 seconds at 10,000 rpm.

- Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for 2 minutes, taking care that the pellets remains in the tube.

Note: In rare cases the pellets may be loose, so pour slowly. Invert the tube onto an absorbent paper minimizes backflow of supernatant from the rim and slides of the tube onto the pellet.

- Add 50μl buffer FG2/QIAGEN® Protease enzyme mixture. Close the tube and vortex immediately until the pellet is homogenized. Inspect the tube to check that homogenization is complete. If traces of pellets with a jelly-like consistency remains, add 30μl buffer FG2 and vortex again.

Note: When processing multiple samples, vortex each tube immediately after addition of buffer FG2/QIAGEN® Protease enzyme mixture.

- Invert the tube 3 times; place it in a heating block or water bath preheated at 65°C for 5 minutes.

Note: The sample changes color from red to olive green, indicating protein digestion.

- Add 50μl isopropanol (100%) and mix thoroughly by inversion until the DNA precipitate becomes visible as threads or a clump.

Note: Complete mixing with isopropanol (100%) is vital to precipitate the DNA and must be checked by inspection.

- Centrifuge for 3 minutes at 10,000 rpm. If the resulting pellets are loose, centrifuge for a longer time or at a higher speed.

- Discard the supernatant and briefly invert the tube onto a clean piece of absorbent paper, taking care that the pellet remains in the tube.

- Add 50μl 70% ethanol and vortex for 5 seconds.

- Centrifuge for 3 minutes at 10,000 rpm.

- Discard the supernatant and briefly invert the tube onto a clean piece of absorbent paper for at least 5 minutes, taking care that the pellet remains in the tube.

- Air-dry the DNA pellet for 5 minutes until all the liquids has evaporated. Avoid over-drying the DNA pellet; this makes it difficult to dissolve.

- Add 100ml buffer FG3, vortex for 5 seconds at low speed and dissolve the DNA by incubating for 5 minutes at 65°C in a heating block or water bath. Prolong the incubation time if the DNA is not completely dissolved.

- Close the sample tube and store at -20°C.

DNA Amplification

Reagent

The LightCycler® FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH) was used for this study.

Reagent content

One pack of the reagent contains the following:

- LightCycler® FastStart enzyme
- LightCycler® FastStart Reaction Mix SYBR Green I
- $MgCl_2$ stock solution, 25mM
- Water, PCR-grade.

Preparation of working reagent

SYBR Green mix

This mix consist of the LightCycler FastStart enzyme and the LightCycler® FastStart Reaction Mix SYBR Green I and it's prepared as follows:

- Place a good quantity of crushed ice into an ice container.
- Remove the LightCycler FastStart enzyme (1a) and the LightCycler® FastStart Reaction Mix SYBR Green I (1b) from the freezer and insert them into the crushed ice.
- Cover the LightCycler FastStart Reaction Mix SYBR Green I (1b) with an aluminum foil to keep out light.
- Remove 1a from the ice and spin down at a low speed for a few seconds to allow the reagent to settle at the bottom of the cap.
- Pipette 10µl of the spinned 1a into 1b and mix by pipetting up and down about five times. Do not vortex.
- A total volume of 74µl SYBR Green mix is obtained and it contains the following: FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP), SYBR Green I dye and $MgCl_2.$
- Insert the SYBR Green mix into the crushed ice and cover with an aluminum foil to keep out light.
- The SYBR Green mix is stable for one week when stored at 2-8ºC.
- Avoid repeated freezing and thawing.

- The kit must be stored at -15 to -25 ℃ through the expiration date as printed on the label.

- 1b and the SYBR Green mix must always be kept in the dark.

- The SYBR Green mix must be stored at 2-8℃ for a maximum of one week.

Primers

The following primers were used for this study:

- Forward primer: PL 1473 F 18 with sequence 5′-TAA CGA ACG AGA TCT TAA- 3′

- Reverse primer: PL 1679 R 18 with sequence 5′- GTT CCT CTA AGA AGC TTT- 3′

(Mangold et al., 2005).

Primer conditions

Forward primer

- 100pmol/μl concentration in 570 μl PCR-grade water or Tris buffer (pH 8)

- 5516 g/mol Molar mass

- 95% purification by HPLC +

Note: These conditions vary and depend on the manufacturers order report that accompanies the primers on delivery.

Reverse primer

- 100pmol/μl concentration in 612 μl PCR-grade water or Tris buffer (pH 8)

- 5465 g/mol Molar mass

- 99% purification by HPLC +

Note: These conditions vary and depend on the manufacturers order report that accompanies the primers on delivery.

Preparation of stock primer solution

Both primers were delivered capped in the lyophilized state. The stock solutions were prepared as follows:

- Centrifuge both caps for a few minutes to enable its contents to settle at the bottom.

- Pipette 570 µl and 612 µl of PCR-grade water into the forward and reverse primers respectively (depend on the manufacturers order report).

Note: For long time storage and better stability, Tris buffer (pH 8) can be used in place of the PCR-grade water.

- Vortex for a few seconds and allow them to stand for some time at room temperature to ensure complete dissolution.
- Label and store at -15 to -25 ºC.

Preparation of stock aliquots

In order to prevent contamination of the primer stocks, aliquots are prepared as follows:

- Label twenty 1.5 ml self capped sterilized eppendorf tubes (ten for each primer stock).
- Pipette 50 µl of the forward primer stock into each of the ten labeled tubes.
- Repeat the above process for the reverse primer stock.
- Store at -15 to -25 ºC

Preparation of primer working solutions

- Remove one each of the forward and reverse primer stock aliquots and thaw.
- Add 450 µl of PCR-grade water to each of the thawed aliquots. The concentration of the working solution now becomes 10pmol/ µl.
- Vortex for a few seconds to ensure the mixture is well mixed.
- Label appropriately and store at 2-8ºC

Controls

Positive control

Four positive controls were used for this study. They are *P. falciparum, P. malariae, P. ovale* and *P. vivax.* These were already prepared DNA samples obtained from the Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg, Germany.

Negative control

The PCR-grade water was used as the negative control for this study.

Preparation of PCR reaction master mix

The PCR reaction master mix is a mixture of all the reagents (SYBR Green mix, Stock $MgCl_2$, forward and reverse primers and PCR-grade water) in their calculated amounts as illustrated in the table below:

Table 2: PCR master mix pipetting scheme

	SYBR Green mix (µl)	Plasmodial DNA (µl)	Stock $MgCl_2$ (µl)	PL 1473 F 18 (µl)	PL 1679 R 18 (µl)	PCR-grade water (µl)
Quantity/Test	2.0	2.0	4.4	1.0	1.0	9.6
Quantity/40 Tests	80.0	80.0	176.0	40.0	40.0	384.0

Procedure

- Fill an ice container with broken ice
- Remove all the reagents from the fridge and insert into the ice. Cover the LightCycler® FastStart Reaction Mix SYBR Green I (1b) with an aluminum foil.
- Label one 1.5 ml self capped sterilized eppendorf tubes as mix.
- Prepare the SYBR Green mix and primer working solutions as described in 3.0.3.7.1 bi and 3.0.3.7.2 d above.

Using 40 tests as a reference:

- Pipette 80 µl of the SYBR Green mix into the tube labeled mix.
- Add 176 µl of the stock $MgCl_2$ .Pipette up and down a few times to mix.
- Add 40 µl each of the forward and reverse primers. Pipette up and down a few times to mix.
- Add 384 µl of the PCR-grade water. Pipette up and down a few times to mix
- Vortex briefly to ensure complete mixing of the reagents.
- Insert the master mix into the ice.
- Label the 0.2 ml self capped sterile tubes from 1 to36 (maximum) to match the DNA sample pathology numbers.
- Arrange these labeled tubes in a sample rack.

- Into each of these tubes, pipette 18 μl of the master mix. Cover each tube after the master mix has been placed inside.
- Add 2 μl of the DNA sample into each matching tube to get a total reaction volume of 20 μl.
- The first four tubes must be for the four positive controls namely: *P. malariae, P. falciparum, P. ovale* and *P. vivax* in that order and the last tube for the negative control.
- The samples are ready for the amplification process.

Precaution:

- The pipette tips **<u>MUST</u>** be changed in between each step even if it's a repetition of the same step.
- Filtered pipette tips must be used to prevent possible DNA contamination.
- Powder free latex gloves must be worm and changed as appropriate throughout the process.

Amplification Process

- The reaction protocol is as outlined below (see table 3):
- Pre-Incubation for activation of FastStart DNA polymerase and denaturation of the DNA.
- Amplification of the target DNA.
- Melting Curve for PCR product identification.
- Cooling the rotor and thermal chamber.

Table 3: Amplification reaction protocol

Analysis Mode	Cycles	Segment	Target Temperature	Hold Time
Pre-incubation	1		95 °C	10 minutes
Amplification	40	Denaturation	95 °C	10 seconds
		Annealing	60 °C Touchdown at 0.5 °C for 11 cycles.	5 seconds.
		Extension	72 °C	20 seconds Measuring fluorescence with green channel at wavelength 470-510nm.
Holding	1	Denaturation	95 °C	2 minutes
	1	Cooling for Dye binding	68 °C	30 seconds
Melting	1	Denaturation	95 °C	2 minutes
		Annealing	60 °C	30 seconds
		Melting	68-90 °C Starts at 68°C, increases at each step by 0.6°C, holds for 5 seconds and measure fluorescence at 470-510nm.	5 seconds each step
Cooling	1		40 °C	20 seconds

APPENDIX 4

Slide preparation

Thick film Preparation

- Using a micro-pipette place 12 μL drop of blood in the larger circle of the slide template on a pre-labeled slide.

- Place the micro-pipette tip in the 12 μL drop of blood and, using a circular motion, spread the blood so that it fills the larger circle or use a second microscope slide or an applicator to spread the blood.

- Air dry the slide on a flat surface. This slow drying avoids cracking.

- If the thick film dries too quickly it may "crack". A dry smear can be easily recognized by holding it to light and noting any wet areas. (Dry slides can then be stored vertically in slide boxes up to 72 hours.)

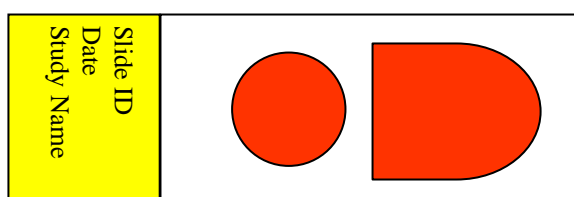- The blood must be well mixed before the films are prepared.
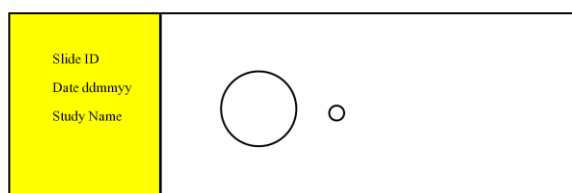
Diameter of thick smear	15mm
Amount of blood for thick smear	12μl
Amount of blood for thin smear	2μl
Area covered by thick blood film	176.78sq.mm

Template for thick and thin blood film preparation
Source: MDCoE, Kisumu-Kenya

Thin film Preparation

- Using a micro-pipette place a 2μL drop of blood in the smaller circle of the slide template. Do not delay between applying the drop and spreading the drop.

- Obtain a second slide and place it in front of the 2µL drop of blood at a 30º - 45º angle. Pull back the slide and hold until the suspension is evenly spread along the width of the spreader slide. Use a spreader slide with a clean and polished end.

- Push the slide forward in a smooth, continuous motion. Avoid hesitation or jerky motions when spreading the blood. (The feathered end of the film should have RBCs that are in one single, distinctive layer).

- To avoid cross contamination, do not re-use the same slide for another subject's blood sample.

Fixing

- Fix the thin film by gently immersing it into absolute methanol (never ethanol) in a Coplin jar. Allow the film to dry naturally in a vertical position. Care must be taken not to accidentally fix any portion of the thick film. The thin film is dipped into methanol and immediately removed.

- Heat fix by blowing hot air (about 45° C) over the slides for 20 – 30 minutes or placing it in a dry box for 40 minutes. This gentle "heat fixation" allows thick films to adhere to the microscope slide much better.

- At all times during preparation and storage, slides should be protected from exposure to insects and dust.

Giemsa staining

Preparation of Giemsa buffer

a. Preparation of buffered water using buffer pellets

- Measure 1000 mL distilled or de-ionized water into a graduated cylinder

- Transfer the 1000 mL distilled or de-ionized water into a buffer bottle.

- Using a forceps or spatula, pick one pellet and put in the buffer bottle.

- Put a magnetic stirring bar into the bottle and close it tightly.

- Place the cylinder on a stirring plate. Allow the contents to stir until the reagents are dissolved completely.

- Check the pH as outlined in (d) below

b. Checking the pH of the buffered water

- Prepare the pH meter in accordance with manufacturer's instruction

- Remove the probe from the store solution, rinse with distilled water and wipe excess water with paper towel.

- Put the probe into the buffer solution and read the pH as displayed on the meter.

- The pH of the buffered water should be between 7.0 and 7.2.

- If the pH of the buffered water is too acidic, add small quantities of the 2% Na_2HPO_4 and recheck with the pH meter. Repeat this process until the desired pH is obtained.

- If the pH of the buffered water is too alkaline, add small quantities of the 2% KH_2PO_4 and recheck with the pH meter. Repeat this process until the desired pH is obtained.

- Store the buffer in a plastic container. The container should be labeled with contents, date prepared, expiration date, and technician initials.

- The buffer is considered expired 7 days after preparation.

Preparation of Giemsa working solution and staining

a. For routine malaria blood film (10% solution) (Working Solution)

- Pour 90 mL of buffered water (pH 7.0- 7.2) into a 100 mL graduated cylinder.

- Using a serological pipette, draw up 10 mL of Giemsa stain. Add the stain to the buffered water in the graduated cylinder.

- Cover the top of the graduated cylinder with Para film or protected hands. Gently invert the cylinder several times (or use a magnetic stirrer) until completely mixed.

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- Label the cylinder with contents, date prepared, time prepared, expiration time, and technician/technologists initials.

- Buffered Giemsa stain (working solution) must be discarded and prepared afresh after 6 hours.

b.   Technique of staining with Giemsa stain solutions

- Each malaria blood film is stained singly on a staining rack, rather than together in batches, to avoid cross-contamination.

- Routine and QC malaria blood films (both thick and thin films) will be stained in 3% Giemsa by flooding the slide (diluted in buffered water of pH 7.0-7.2) for 45-60 minutes.

- Acute malaria and quality control blood films (both thick and thin films) will be stained in 10% Giemsa by flooding the slide for 10-15 minutes.

- Rinse the slide briefly and gently by gentle running tap water or by a gentle flow of clean water from a beaker.

- Let the slides dry in a vertical position. (Drying may be hastened by use of a blow drier or slide warmer).

- Keep the slides in the slide box/folder in sequential order according to subject identification numbers.

- At all times during preparation and storage, slides should be protected from exposure to insects and dust.

Reading and quantification of parasites

a.   Quantification of parasites in thick films

- The following method was used for quantifying asexual *Plasmodium* forms (in either single or mixed species infections) as well as sexual (gametocyte) forms. (If different species are observed, this fact will also be recorded).

- Piano-type tally counters will be used for counting asexual parasite forms and for counting WBCs.

- If parasites are observed, count them while simultaneously counting WBCs, up to a total of 200 WBCs. (But ensure that all parasites in the final HPF are counted even if a count of 200 WBCs has been exceeded.)

- A malaria blood film was considered negative if 100 HPFs have been scanned and no parasite observed.

- A recent laboratory WBC count is used to convert a parasite count to a parasite density (per µl of blood) by the following formula:# Parasites x (WBCs per µl blood) / # WBCs = Parasites/µl

- If the parasite/field exceeds 100 in a thick film, discontinue the thick film count and switch to the thin film instead.

b. **Quantification of infected RBCs in Thin films**

- Identify an area in the thin film where RBCs do not overlap, preferably in the tail (feathered edge) of the thin film.

- Upon observation of malaria parasites, begin to count parasitized RBCs per 1,000 total RBCs.

- Perform the count across the width of the thin film using the "battlement method" and stop the count on the 1000th RBC.

- *After the first reading, slides should be kept in the same order in the slide box/folder for the second reader (who will follow the same procedure, but will record the results on a different Microscopist work sheet).*

- Acute slides are read in an expedited fashion. This system is used ONLY to guide clinical management of subjects, whereas parasite densities are used to determine final results.

- Record all the HPFs scanned, parasites counted, WBCs counted, and parasitized RBCs counted into the "Microscopist Worksheet" or the Malaria Microscopy Logbook provided.

Tuesday, July 27, 10:00 am – 12:30 pm

Tuesday AM, July 27

Poster Session: 10:00 am – 12:30 pm
Infectious Disease

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Addressing the challenges of the Giemsa stain in the diagnosis of malaria in an endemic area using the Partec CyScope[∗].

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Introduction One of the main interventions of the Global Malaria Control Strategy is the prompt and accurate diagnosis of malaria as it is the key to effective disease management (A global strategy for malaria control, 1993). It is thus of concern that poor diagnosis continues to hinder effective malaria control. The laboratory diagnosis of malaria has up to now relied nearly exclusively on microscopy, a valuable technique when performed correctly but unreliable and wasteful when poorly executed. A better utilization of microscopy and the development of alternative diagnostic techniques could substantially improve malaria control (Institute of Medicine, 1991).

Aim To evaluate the Partec CyScope® (PC) for the diagnosis of malaria in human blood from patients in an endemic area using an expanded gold standard.

Materials and Methods 263 samples from patients attending the Under Five Clinic and OPD of the Agogo Presbyterian Hospital in the Asante Akim North District, Ghana were examined with two different tests independently and blinded. The test methods employed in the study were: (i) Giemsa-stained blood smears (GS) (ii) PC

Outcome 107 (40.7%), 111 (42.2%) samples were positive for the presence of Plasmodia by GS and PC respectively. Compared to the expanded gold standard the sensitivity of GS and PC was 94.7% and 95.6% at 95% confidence interval. The specificity was 100% and 98% at 95% confidence interval. The positive predictive value was 100% and 97.3% and the negative predictive value was 96.2% and 96.7% respectively with good agreements k=0.95 and 0.94. The test performance of PC was very similar to GS. However, the PC had added advantages; it was faster, easier to use, less expensive in terms of cost per test and test equipment than the GS and has a standby battery making it ideal for field work and places where there is no electricity. The PC can therefore be used as an alternative method for GS.